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# Liquid Chromatographic Separation of Isoniazid, Pyrazinamide and Rifampicin on a Reversed-Phase Silica Column

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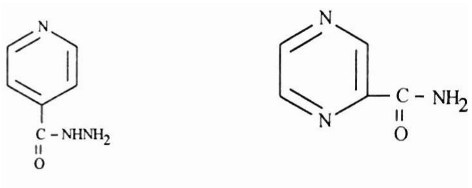
**A gradient liquid chromatographic method which can separate isoniazid, pyrazinamide and rifampicin is described. A Hypersil $C_{18}$, 5 µm, 250 mm x 4.6 mm internal diameter column was maintained at 40°C. The method was developed by systematic evaluation of the influence of the buffer concentration, column temperature and the mobile phase pH. The method proposed uses isocratic elution with potassium phosphate buffer (pH 6.0; 0.05 M) for 10 min, followed by linear gradient to potassium phosphate buffer (pH 6.0; 0.05 M)-methanol (40:60, v/v) in 5 min, isocratic elution at the same composition for a further 15 min and then linear gradient back to potassium phosphate buffer (pH 6.0; 0.05 M) in 5 min. The flow-rate was 1 ml/min and UV detection was at 254 nm. The method was validated and it has been used for routine analysis of tablets containing isoniazid, pyrazinamide and rifampicin. Analysis time is 35 minutes.**

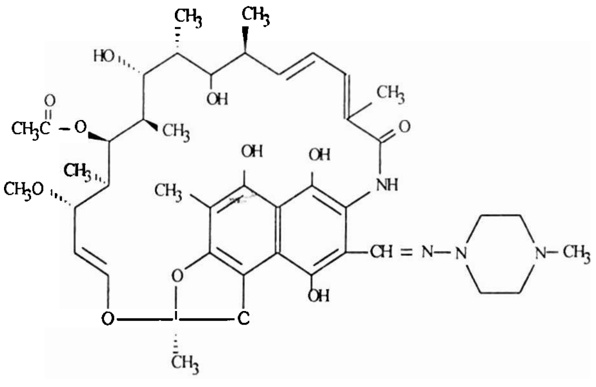
**Key words:** Reversed-phase chromatography, gradient, isoniazid, pyrazinamide, rifampicin, gradient.

## INTRODUCTION

Tuberculosis (TB), a disease caused by *Mycobacterium tuberculosis*, has been declared a global emergency by the World Health Organisation. It is currently killing about two million people and eight million more are infected annually. Besides, it is also often associated with acquired immune deficiency syndrome (AIDS). Short course chemotherapy is the first choice recommended regimen for new sputum smear positive pulmonary TB patients, new sputum smear negative or extra-pulmonary patients who are in very bad clinical condition and for new patients with miliary TB, TB meningitis or a recent TB spondylitis without deformity. This regimen consists of an intensive phase, during which the patient is treated daily for two months with streptomycin, isoniazid (INH), pyrazinamide (PZA) and rifampicin (RIF) under medical supervision, followed by the continuation phase during which the patient takes thiacetazone and isoniazid daily at home for another six months [1]. To improve compliance, many pharmaceutical companies produce isoniazid, pyrazinamide and rifampicin (Figure 1) in a fixed-dose combination tablet.



**Isoniazid** **Pyrazinamide**



**Rifampicin**

**Figure 1: Structures of isoniazid, pyrazinamide and rifampicin**

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Analysis of this combination tablet is time-consuming because official compendia give different methods for the active ingredients. The European Pharmacopoeia (Ph. Eur.) gives methods for analysis of the raw materials only [2], while the British Pharmacopoeia (B.P.) gives different methods for analysis of each of the three active ingredients in a single-dose formulation [3]. The United States Pharmacopoeia (U.S.P.) recommends a liquid chromatographic method for analysis of rifampicin, which is different from that for isoniazid and pyrazinamide [4].

Various workers have developed LC methods for simultaneous assay of isoniazid and pyrazinamide [5-9] and for isoniazid and rifampicin [10-14] separately. Spectroscopic methods have been used for simultaneous assay of the three drugs [15,16]. Gaitonde *et al.* used an Excalibar ODS-CN 5 µm column and methanol-tetrabutylammonium hydroxide pH 3.0 (80:20, v/v) as the mobile phase to separate the three compounds with UV-detection at 254 and 265 nm [17]. In this system, pyrazinamide eluted at the solvent front. Woo *et al.* reported the use of Hibar-LiChrosorb RP-8, 5 µm with detection at 215 nm using a gradient system containing acetonitrile and 10 mM phosphate buffer [18]. Isoniazid and pyrazinamide were not well separated. Rao *et al.* used a Porasil 5 µm column and a mobile phase consisting of isopropanol-methanol-potassium hydrogen phosphate (0.136 %) (56:42:2.5, v/v) to separate isoniazid, pyrazinamide and rifampicin [19].

This paper reports the development of an LC method for simultaneous assay of isoniazid, pyrazinamide and rifampicin using a more commonly used column as well as less expensive solvents.

## EXPERIMENTAL

### Samples and reagents

Isoniazid, pyrazinamide and rifampicin drug substances were from B.D.H. Chemicals (Poole, England), Novochem (Hamburg, Germany) and British Pharmacopoeia (London, U.K.), respectively. Tablets containing INH 50 mg, PZA 300 mg and RIF 120 mg were from Universal Pharmacy Ltd (Nairobi, Kenya) and Gruppo Lepetit (Milan, Italy), those containing INH 150 mg and RIF 300 were from Novartis Pharma AG (Basle, Switzerland) and Hoechst Marion Roussel (Midrand, South Africa), while tablets containing INH 300 mg and RIF 450 were from Lupin Laboratories Ltd (Chikalthana, India). Liquid chromatography-grade methanol from Rathburn (Walkerburn, Scotland) was used. Water was distilled in glass apparatus. All other reagents were of pro-analysi quality (Acros, Beerse, Belgium).

### Sample preparation

Samples for method development were prepared by accurately weighing 10 mg INH, 10 mg PZA and 24 mg RIF drug substances into a 25.0 ml volumetric flask. About 5 ml of methanol was added and the contents sonicated for 5 minutes, made to volume with potassium phosphate buffer (pH 6.0; 0.05 M), mixed thoroughly and then filtered through membrane filter (0.45 µm). Test solutions were prepared similarly using tablet powder equivalent to about 10 mg of INH.

### Liquid chromatography

Liquid chromatographic (LC) apparatus consisted of Merck-Hitachi L-6200A (Darmstadt, Germany) solvent delivery system, used at a flow rate of 1 ml/min, an injector Model 725i (Rheodyne, L.P., Cotati, CA, U.S.A.) equipped with a 20 µl loop, a Merck-Hitachi Model L-4200 variable UV detector set at 254 nm and a Hewlett-Packard Model 3396 Series II integrator (Avondale, PA, U.S.A.). The stationary phases examined were Hypersil $C_{18}$ 5 µm (Shandon, Cheshire, England), RSil $C_{18}$ 5 µm (Alltech, Deerfield, IL, U.S.A.), PRP-1 7-9 µm (Hamilton, Reno, NV, U.S.A.), Rogel 80 Å 8 µm (Bio-Rad, Eke, Belgium), Zorbax $C_8$ (DuPont, Wilmington, DE, U.S.A.), PLRP-S 100 Å 8 µm, PLRP-S 300 Å 8 µm and PLRP-S 1000 Å 8 µm (Polymer Laboratories, Church Stretton, Shropshire, U.K.), all in 250 mm x 4.6 mm I.D. columns and equilibrated by means of a water-bath. The method uses isocratic elution with potassium phosphate buffer (pH 6.0; 0.05 M) for 10 min, followed by linear gradient to potassium phosphate buffer (pH 6.0; 0.05 M)-methanol (40:60, v/v) in 5 min, isocratic elution at the same composition for a further 15 min and then linear gradient back to potassium phosphate buffer (pH 6.0; 0.05 M) in 5 min.

**Table 1: Chromatographic separation parameters of isoniazid and pyrazinamide on different stationary phases**

| Column | Capacity Factor | | Peak Symmetry | | Resolution | Plate Number | |
|---|---|---|---|---|---|---|---|
| | INH | PZA | INH | PZA | INH/PZA | INH | PZA |
| Hypersil $C_{18}$ 5 µm | 1.2 | 1.8 | 2.2 | 2.0 | 3.5 | 3035 | 3670 |
| PLRP-S 100 Å 8 µm | 0.9 | 2.3 | 5.1 | 1.9 | 3.7 | 660 | 1000 |
| PLRP-S 300 Å 8 µm | 1.2 | 2.8 | 3.4 | 2.5 | 3.9 | 570 | 990 |
| PRP-1 7-9 µm | 1.2 | 2.4 | 4.9 | 1.8 | 2.8 | 320 | 400 |
| PLRP-S 1000 Å 8 µm | 0.3 | 0.9 | A | a | 1.6 | 150 | 570 |
| RSil C18 5 µm | 6.5 | 5.7 | B | b | b | b | b |
| Zorbax $C_8$ | 1.8 | 2.3 | B | b | b | b | b |

**Mobile phase: potassium phosphate buffer (pH 6.0; 0.05 M). Column temperature: 50 °C. Detection: UV at 254 nm. a: No separation of isoniazid from pyrazinamide at 0.05 of peak height. b: No separation of isoniazid from pyrazinamide at 0.5 of peak height.**

## RESULTS AND DISCUSSION

### Development of the liquid chromatographic method

The structures of INH and PZA are quite similar, thus resulting in separation problems. The initial step in developing this method involved search of a suitable stationary phase to separate a mixture of INH from PZA. All columns were maintained at 50 °C to increase efficiency and reduce backpressure. Inclusion of an organic modifier in the mobile phase effected no separation and so potassium phosphate buffer was used as the mobile phase. Preliminary studies were done at pH 3.0, 6.0 and 10.0 for the polymer columns and at pH 3.0 and 6.0 for the silica based columns. For the polymer columns pH had little effect on selectivity, but for the silica columns there was a slight decrease in retention of INH upon decreasing the buffer pH. Table 1 shows the chromatographic parameters of separation for INH and PZA using different columns, which were calculated using equations from the Ph. Eur. [2]. Good resolution was obtained using Hypersil $C_{18}$ 5 µm, PLRP-S 100 Å 8 µm, PLRP-S 300 Å 8 µm and PRP-1, 7-9 µm columns. However, the polymer columns had very low efficiency and produced very asymmetric INH peaks, and so Hypersil $C_{18}$ 5 µm was chosen for further work.

There was little change in chromatographic parameters when the buffer concentration was varied between 0.03 M and 0.07 M (table 2). Small changes in ionic strength have little effect on the retention. A concentration of potassium phosphate buffer (0.05 M) was found suitable.

**Table 2: Influence of concentration of the buffer on the chromatographic separation parameters of isoniazid and pyrazinamide**

| x | Capacity Factor | | Peak Symmetry | | Resolution |
|---|---|---|---|---|---|
| | INH | PZA | INH | PZA | INH/PZA |
| 0.03 M | 1.2 | 1.8 | 2.0 | 1.9 | 3.5 |
| 0.05 M | 1.2 | 1.8 | 2.2 | 2.0 | 3.5 |
| 0.07 M | 1.2 | 1.8 | 2.0 | 2.0 | 3.3 |

**Mobile phase: potassium phosphate buffer (pH 6.0; x M). Flowrate: 1 ml/min. Column: Hypersil $C_{18}$ 5 µm. Column temperature: 50 °C. Detection: UV at 254 nm.**

Having achieved separation of INH and PZA, further investigations focused on separating a mixture of INH, PZA and RIF. Rifampicin (RIF) is strongly retained on reversed-phase silica and it necessitated the introduction of an organic modifier after elution of INH and PZA. Methanol was used in the following gradient elution: potassium phosphate buffer (pH 6.0; 0.05 M) for 10 min, changed by linear gradient to buffer (pH 6.0; 0.05 M)-methanol (40:60, v/v) in 5 min, held at the same composition for a further 15 min and then changed back to potassium phosphate buffer (pH 6.0; 0.05 M) in 5 min. The system was held at 100 % of potassium phosphate buffer (pH 6.0; 0.05 M) for 5 minutes before the next injection. Using this elution good separation was achieved.

**Table 3: Influence of column temperature on the chromatographic separation parameters of isoniazid, pyrazinamide and rifampicin**

| Temperature | Capacity Factor | | | Resolution INH/PZA | Peak Symmetry | | |
|---|---|---|---|---|---|---|---|
| | INH | PZA | RIF | | INH | PZA | RIF |
| 30 °C | 3.3 | 4.8 | 10.4 | 6.7 | 1.8 | 1.6 | 1.9 |
| 40 °C | 2.0 | 3.1 | 7.2 | 3.9 | 1.4 | 1.4 | 1.5 |
| 50 °C | 1.0 | 1.8 | 7.1 | 3.8 | 1.3 | 1.4 | 1.6 |

**Mobile phase: potassium phosphate buffer (pH 6.0, 0.05 M) for 10 min, up to potassium phosphate buffer (pH 6.0; 0.05 M)-methanol (40:60, v/v) in 5 min, held for a further 15 min and then back to potassium phosphate buffer (pH 6.0, 0.05 M) in 5 min. Flowrate: 1 ml/min. Column: Hypersil $C_{18}$ 5 µm. Detection: UV at 254 nm**

When column temperature was decreased, there was an increase in retention of all the analytes (table 3). RIF was well resolved from INH and PZA. A temperature of 40 °C was chosen because it gave good balance between resolution, retention and peak symmetries.

Finally, the effect of mobile phase pH was investigated further (table 4). Table 5 shows $pK_a$ values of the compounds under study [20,21]. Basic compounds show strong retention and peak tailing on silica-based columns due to hydrogen bonding and/or ion exchange with silanol groups [22]. In aqueous solutions, acidic silanol groups ($pK_a$ 7.1) behave as cation-exchangers at pH 2-6. Below pH 2 (isoelectric state) they should act as anion exchangers, while above pH 9 these groups are largely deprotonated [23]. The first protonation of INH ($pK_a$ 2.0) occurs at the pyridine nitrogen and the second ($pK_a$ 3.6) at the hydrazine group [21]. At pH 3, INH is highly protonated and it interacts with the silanol groups mainly by cation-exchange, thereby tailing strongly. With increase in pH there is less protonation and hence less tailing as observed at pH 4-7. At pH 3, retention is low because of high polarity and hence less interaction with the hydrophobic reversed phase. Retention is constant between pH 4 and pH 6 where the molecule is mainly neutral and then there is a slight decrease in retention at pH 7, because of decrease in cation-exchange. Pyrazinamide is neutral throughout the pH range studied and therefore its retention is constant from pH 3 to pH 6. Similarly to INH, at pH 7 there is a slight decrease in retention. It interacts less with silanol groups and so it tails less than INH. Rifampicin has a deprotonated phenolic group and a protonated amino function throughout the pH range studied. At pH 3.0, RIF is strongly retained possibly because of reduced polarity, as the phenolate group is partly protonated.

**Effect of mobile phase pH on chromatographic separation parameters of isoniazid, pyrazinamide and rifampicin.**

| X | Capacity Factor | | | Resolution INH/PZA | Peak Symmetry | | |
|---|---|---|---|---|---|---|---|
| | INH | PZA | RIF | | INH | PZA | RIF |
| 3.0 | 0.9 | 3.1 | 8.3 | 4.9 | 3.7 | 1.5 | a |
| 4.0 | 1.7 | 3.1 | 6.6 | 3.9 | 2.2 | 1.5 | 1.4 |
| 5.0 | 1.8 | 3.0 | 6.8 | 3.5 | 1.4 | 1.4 | 1.6 |
| 6.0 | 2.0 | 3.1 | 7.2 | 3.9 | 1.4 | 1.4 | 1.5 |
| 7.0 | 1.5 | 2.5 | 7.1 | 2.6 | 1.2 | 1.3 | 1.3 |

**Mobile phase: potassium phosphate buffer (pH X; 0.05 M) (0-10 min), up to potassium phosphate buffer (pH X; 0.05 M)-methanol (40:60, v/v) in 5 min, held for a further 15 min and then back to potassium phosphate buffer (pH X, 0.05 M) in 5 min. Column: Hypersil $C_{18}$ 5 µm. Column temperature: 40 °C. Detection: UV at 254 nm. a: No return to baseline.**

**Table 5. $pK_a$ values of isoniazid, pyrazinamide and rifampicin**

| Compound | Isoniazid | Pyrazinamide | Rifampicin | |
|---|---|---|---|---|
| $pK_a$ | $HB^+$ | $HB^+$ | HA | $HB^+$ |
| | $2.0^{c,d}$,<br>$3.5^{c}(3.6^{d})$,<br>$10.8^{c,d}$ | $0.5^{c}$ | $1.7^{c}$ | $7.9^{c}$ |

**c: From ref [20], d: From ref [21]**

Although at pH 7 good peak symmetries were produced, the stability of the column is less than at pH 6. The best mobile phase therefore consisted of potassium phosphate buffer (pH 6.0; 0.05 M) for 10 min, changed by linear gradient to potassium phosphate buffer (pH 6.0; 0.05 M)-methanol (40:60, v/v) in 5 min, held at the same composition for a further 15 min and then changed back to potassium phosphate buffer (pH 6.0; 0.05 M) in 5 min. With this system, RIF eluted within 30 minutes. Figure 2 shows a typical chromatogram.

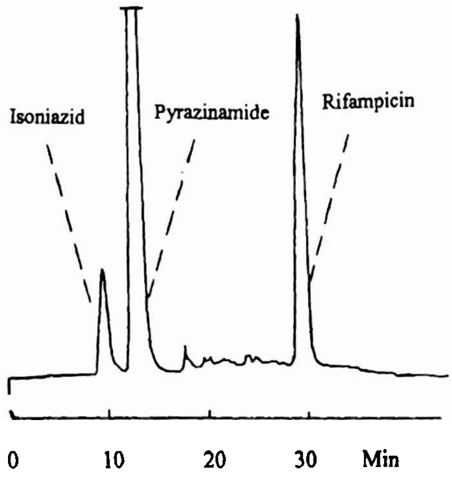

**Figure 2: Liquid chromatogram of isoniazid, pyrazinamide and rifampicin**

**Mobile phase: potassium phosphate buffer (pH 6.0; 0.05 M) for 10 min, changed by linear gradient to potassium phosphate buffer (pH 6.0; 0.05 M)-methanol (40:60, v/v) in 5 min, held at the same composition for a further 15 min and then changed back to potassium phosphate buffer (pH 6.0; 0.05 M) in 5 min. Column: Hypersil $C_{18}$ 5 µm. Column temperature: 40 °C. Detection: UV at 254 nm.**

## Validation of the liquid chromatographic method

Robustness of the method was studied by evaluating effect of small changes in the operating conditions (variables) of the analytical procedure on measured responses. The variables studied were column temperature, concentration of the potassium phosphate buffer, concentration of the organic modifier, the pH and flow rate of the mobile phase. The measured response was the retention time of INH, PZA and RIF and these are given in table 6. Variation of column temperature and concentration of the potassium phosphate buffer had little effect on retention of the analytes. The amount of methanol had little influence on the retention of INH and PZA, because these two compounds elute before methanol is introduced into the system, but it had a big influence on the retention of RIF. The pH and the flow-rate of the mobile phase had the greatest influence on retention of all three compounds. However, these changes have little effect on the resolution of the compounds and the method remains selective over the range of conditions examined. The method is therefore robust for the simultaneous analysis of isoniazid, pyrazinamide and rifampicin.

**Table 6. Influence of column temperature, concentration of the potassium phosphate buffer, concentration of the organic modifier, the pH and flow-rate of the mobile phase on retention times of isoniazid, pyrazinamide and rifampicin.**

| Variable | | Retention | time | |
|---|---|---|---|---|
| | | Isoniazid | Pyrazinamide | Rifampicin |
| 1. Column temperature (°C) | 38 | 11.0 | 13.0 | 30.4 |
| | 40 | 10.4 | 13.8 | 29.7 |
| | 42 | 10.0 | 13.5 | 30.1 |
| 2. Buffer pH 6.0 (M) | 0.04 | 10.3 | 13.7 | 29.7 |
| | 0.05 | 10.4 | 13.8 | 29.7 |
| | 0.06 | 10.1 | 13.7 | 27.4 |
| 3. Methanol (v/v) | 58 | 10.4 | 14.0 | 33.6 |
| | 60 | 10.4 | 13.8 | 29.7 |
| | 62 | 10.4 | 13.9 | 27.7 |
| 4. pH | 5.8 | 10.1 | 13.1 | 27.1 |
| | 6.0 | 10.4 | 13.8 | 29.7 |
| | 6.2 | 9.2 | 12.6 | 21.0 |
| 5.Flow rate (ml/min) | 0.9 | 11.7 | 15.5 | 31.1 |
| | 1.0 | 10.4 | 13.8 | 29.7 |
| | 1.1 | 9.5 | 11.2 | 29.0 |

**Table 7. Validation parameters for isoniazid, pyrazinamide and rifampicin**

| Parameter | Isoniazid | Pyrazinamide | Rifampicin |
|---|---|---|---|
| 1. Linearity of detector response | | | |
| Y | 39.989x + 0.030 | 37.173x + 4.703 | 50.751x + 9.819 |
| R | 0.9996 | 0.9974 | 0.9976 |
| Standard error of estimate | 0.151 | 2.599 | 0.131 |
| Standard error of slope | 0. 278 | 1.109 | 0.723 |
| Standard error of intercept | 0.110 | 0.962 | 1.692 |
| Concentration range (mg/ml) | 0.16-0.56 | 0.96-3.36 | 0.38-1.34 |
| 2. Precision (n=6) | | | |
| Concentration used (mg/ml) | 0.40 | 2.40 | 0.96 |
| Within-day RSD (%) | 0.53 | 1.07 | 0.56 |
| 3. Limit of detection (S/N=3) (ng) | 0.20 | 0.02 | 0.004 |
| 4. Limit of quantitation (S/N=10) (ng) | 1.00 | 0.04 | 0.02 |
| 5. Stability | | | |
| a) in solution at 25 °C (h) | 6 | 25 | 25 |
| Degradation (%) | 1.69 | 0.31 | 0.88 |
| b) in column at 40 °C (min) | 15 | 15 | 40 |
| Degradation (%) | 1.38 | 0.14 | 0.36 |

**y: Chromatographic peak area (x $10^{-6}$). X: concentration in mg/ml. R: correlation coefficient. RSD: relative standard deviation . S/N: signal to noise ratio**

**Table 8. Composition of combination antitubercular drugs as a percentage (m/m) of the label claim**

| | | Assay results in % m/m ( Label Claim) | | |
|---|---|---|---|---|
| **Product** | **Age (months)** | **INH** | **PZA** | **RIF** |
| A | 24 | 103.9% (50 mg) | 102.1% (300 mg) | 100.1% (120 mg) |
| B | 16 | 96.6% (50 mg) | 100.6% (300 mg) | 105.6% (120 mg) |
| C | 24 | 101.1% (300 mg) | - | 112.9% (450 mg) |
| D | 20 | 100.3% (150 mg) | - | 92.2% (300 mg) |
| E | 40 | 101.6% (150 mg) | - | 101.1% (300 mg) |

Table 7 gives the validation parameters for the method under study. The linearity of detector response was determined at 254 nm by injecting (n=3) solutions corresponding to 40 %, 60 %, 80 %, 100 %, 120 % and 140 % of the sample concentration in the test solution (0.4, 2.4 and 0.96 mg/ml corresponds to 100 % of INH, PZA and RIF, respectively). The method was found to be linear. The method was assessed by analyzing solutions at 100 % concentration and it was found to be repeatable. The limit of detection and limit of quantitation were determined at a signal to noise ratio of 3 and 10, respectively. The analytes showed no degradation in solution when stored at 4-8 °C for 25 h and showed slight degradation when kept for more than 6 h in the dark at 25 °C. Stability of INH and PZA in the column was estimated by keeping a solution of the drugs in potassium phosphate buffer (pH 6.0; 0.05 M) at 40 °C for 15 min. Rifampicin, which is not soluble in the buffer and does not elute with the buffer alone, was first dissolved in 6 ml methanol and then 4 ml of potassium phosphate buffer (pH 6.0; 0.05 M) was added before stressing it at 40 °C for 40 min . The amount of degradation was calculated from the difference between chromatographic areas of the stressed solutions and that of the unstressed ones. All three drugs were found to be stable under conditions similar to those in the column.

The method was used to analyze commercial samples which were run against standard mixtures and some results are shown in table 8. The content of all the products analyzed lies within the U.S.P. specifications for the combined drugs.

## AKNOWLEGEMENT

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