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High-performance liquid chromatographic determination of the 1,4-diketo and 1,4-diketo-2,3-dihydroxy metabolites of lonapalene in rat urine

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ABSTRACT

Individual high-performance liquid chromatographic (HPLC) methods have been developed for the determination of two major metabolites of lonapalene in rat urine. The highly unstable and polar 1,4-diketo-2,3-dihydroxy metabolite (II) is extracted from urine by two extraction columns (phenyl followed by silica), further purified by means of HPLC with a fully end-capped C_{18} HPLC column and quantified by an ultraviolet detector at 280 nm. Ascorbic acid is used as an antioxidant during extraction and overnight injection of II. Urine samples for total II (free plus conjugated) determination are incubated with arylsulfatase and β-glucuronadase prior to extraction. The 1,4-diketo metabolite (III) is extracted from urine with a C_{18} extraction column, further purified with a C_{18} HPLC column, and quantified by an ultraviolet detector at 260 nm. The detection limit for both metabolites is 100 ng/ml of urine (signal-to-noise = 2.5). The methods were used to analyze urine samples from a long-term toxicology study of lonapalene in rats and to determine the linearity of dose–concentration relationships for both metabolites.

INTRODUCTION

Lonapalene (6-chloro-1,4-diacetoxy-2,3-dimethoxynaphthalene, I, Fig. 1), a novel antipsoriatic agent with rapid topical efficacy and minimal undesirable effects [1–3], is now under investigation at Syntex. Recent metabolism studies [4,5] have shown that 17% of the topically applied lonapalene is absorbed by rats and is rapidly metabolized systematically. Detection of its presence in plasma or urine is difficult. However, after dermal administration of [^{14}C]lonapalene to the backs of male rats, two of the major metabolites found in rat urine have been identified by chromatographic retention times as 1,4-diketo-2,3-dihydroxylonapalene (II, Fig. 1) and 1,4-diketolonapalene (III, Fig. 1). Enzymatic hydrolysis of urine samples further revealed that II exists largely in conjugated forms [5]. Determination of the concentrations of these two metabolites in urine provides valuable information about the systemic metabolism of lonapalene, and also provides indirect evidence about systemic absorption of lonapalene in long-term toxicology studies in animals.

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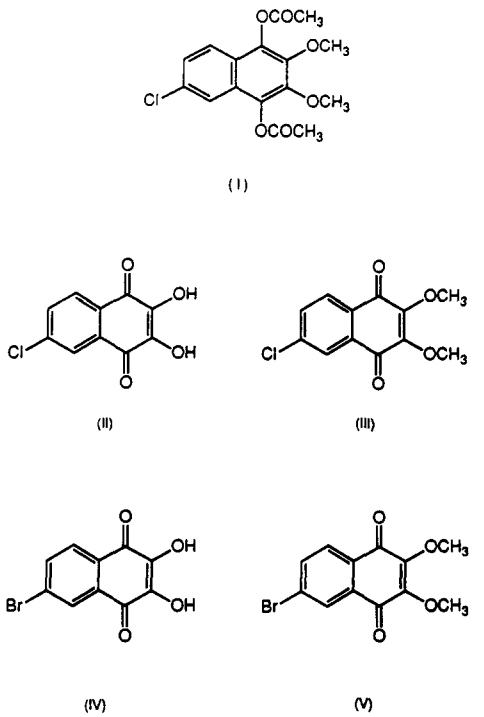

Fig. 1. Structures of lonapalene (I), metabolite II, metabolite III, internal standard IV (RS-90761), and internal standard V (RS-28606).

Because of the very large differences in polarity and stability between these two metabolites, it was necessary to develop a separate procedure for quantitative extraction of each one from biological fluids. The two metabolites also had different optimal detection wavelenghts. We, therefore, chose to develop separate but simple isocratic high-performance liquid chromatographic (HPLC) assay methods for each metabolite. The methods were used to determine the concentrations of these two metabolites in rat urine samples from a longterm dermal carcinogenicity study. In this report we present the HPLC methods for the determination of II and III in urine, along with some of the analytical results obtained in the carcinogenicity study.

EXPERIMENTAL

Chemicals and reagents

Acetonitrile, methanol and tetrahydrofuran (THF) were HPLC grade and purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.); tetrabutylammonium phosphate and ascorbic acid were from Kodak (Rochester, NY, U.S.A.); citric acid and sodium acetate were from Mallinckrodt (McCraw Park, IL, U.S.A.);

sulfatase (crude solution from Helix pomatia containing arylsulfatase and β-glucuronidase) was purchased from Sigma (St. Louis, MO, U.S.A.); lonapalene (I), metabolite II (RS-95726), metabolite III (RS-43535), internal standard IV (RS-90761) and internal standard V (RS-26808) (Fig. 1) were obtained from the Institute of Organic Chemistry (Syntex Research, Palo Alto, CA, U.S.A.).

Preparation of standard solutions

Stock solutions (100 μg/ml) of II and of III were each prepared in acetonitrile and stored in 15-ml polypropylene culture tubes. From the corresponding stock solutions, standard solutions of II at concentrations of 2000, 1000, 500, 250 and 125 ng per 100 μl and of III at concentrations of 2000, 1000, 500, 300, 200, and 100 ng per 100 μl were prepared in a mixture of acetonitrile–water (1:1, v/v). All standard solutions were prepared in 15-ml polypropylene culture tubes immediately before each assay. Internal standard solutions for both compounds (IV for the determination of II and V for the determination of III) were each prepared at a concentration of 100 μg/ml in acetonitrile and stored in 15-ml polyprolylene culture tubes.

Enzymatic hydrolysis

For the determination of total (free + conjugated) II, urine samples were subjected to enzymatic hydrolysis prior to extraction. To ensure that the concentrations of the analyte to be determined were always within the linear range of the method, a suitable volume of each sample (between 0.1 and 1.0 ml) was used for the analysis. To a 15-ml polypropylene culture tube were added 0.1–1.0 ml of a urine sample, 0.5 ml of freshly prepared 1.0 *M* ascorbic acid, 1.0 ml of 0.2 *M* acetate buffer at pH 5.0, 10 μl of internal standard solution, and 0.5 ml of sulfatase solution containing 1000–2500 U[a] of sulfatase and approximately 50 000 U[b] of glucuronidase. The mixture was vortex-mixed (Thermolyne Mixer, Dubuque, IA, U.S.A.) for a few seconds and incubated at room temperature for 1 h. After the incubation, the mixture was subjected to extraction (see below).

Extraction and HPLC quantification of 1,4-diketo-2,3-dihydroxylonapalene

All reversed-phase extraction columns were washed before use with 1.0 ml of methanol and then equilibrated with 1.0 ml of water. A suitable volume of urine sample (0.1–1.0 ml) or 1.0 ml of blank urine sample spiked with 100 μl of a standard solution was added to a 15-ml polypropylene culture tube containing 1.0 ml of 1.0 *M* ascorbic acid and 10 μl of internal standard solution. The mixture was vortex-mixed for a few seconds. The mixed solution obtained here or from enzymatic hydrolysis described above was passed through a 150-mg phenyl ex-

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[a] One unit will hydrolyze 1.0 μmol of *p*-nitrocatechol sulfate per h at pH 5.0 at 37°C (30-min assay).

[b] One unit will liberate 1.0 μg of phenolphthalein from phenolphthalein glucuronic acid per h at pH 3.8 at 37°C (30-min assay).

traction column with the aid of gravity. The column was made by packing 40-μm phenyl packing material (Analytichem International, Harbor City, CA, U.S.A.) into a 5-ml empty polyethylene column equipped with 20-μm frit (E & K Scientific, Saratoga, CA, U.S.A.). After the initial extraction, the column was rinsed with 1.0 ml of water. The analyte was then eluted with 1.0 ml of acetonitrile. The eluate in acetonitrile was passed through a 150-mg silica extraction column previously washed with 1.0 ml of methanol followed by 1.0 ml of acetonitrile. The silica column bed was rinsed with 1.0 ml of acetonitrile and the analyte was then eluted with 0.5 ml of 0.1 *M* citric acid containing 0.1 *M* ascorbic acid. A suitable aliquot of the eluate was injected onto a fully end-capped C_{18} column (BDS Hypersil, 5 μm, 150 mm x 4.6 mm I.D., Keystone Scientific, Bellefonte, PA, U.S.A.) by an autoinjector (Water 710B, Waters Assoc., Milford, MA, U.S.A.). The mobile phase (acetonitrile–THF–0.01 *M* citric acid and 0.01 *M* tetrabutylammonium phosphate, 12.5:12.5:75 v/v) eluted the analyte through the column at a flow-rate of 1.0 ml/min with the aid of an HPLC pump (Rabbit HPX, Rainin Instrument, Emeryville, CA, U.S.A.). The effluent was monitored with a UV detector (Spectroflow 783, Kratos Division, ABI, Ramsey, NJ, U.S.A.) at 280 nm. The data generated by the detector were acquired, processed and quantified by a laboratory data system (Nelson 6000, Nelson Analytical, Cupertino, CA, U.S.A.).

Extraction and HPLC quantification for 1,4-diketolonapalene

To a 15-ml polypropylene culture tube were added 10 μl of internal standard solution, 1.0 ml of water, a suitable volume of a urine sample (0.1–1.0 ml) or 1.0 ml of blank urine sample spiked with 100 μl of a standard solution. The mixture was vortex-mixed for a few seconds and passed through a 100-mg C_{18} extraction column (E&K Scientific) with the aid of gravity. The column bed was rinsed with 0.5 ml of water followed by 1.0 ml of 30% methanol in water. The analyte was eluted with 1.0 ml of methanol. After dilution with 1.0 ml of water, 0.2 ml of the eluate was injected by an autoinjector (Waters 710B) onto a C_{18} HPLC column (Partisil ODS-3, Phenomenex, Terrace, CA, U.S.A.). A mobile phase consisting of acetonitrile– and 0.01 *M* citric acid (53:47) was delivered by an HPLC pump (Rabbit HPX) at a flow-rate of 1.0 ml/min to elute the analyte through the column. A UV detector (Spectroflow 783) was used to monitor the effluent at 260 nm. The same data collection and processing used for quantification of II was used for III.

RESULTS AND DISCUSSION

Stability and adsorption of 1,4-diketo-2,3-dihydroxylonapalene

II, the dihydroxy metabolite of lonapalene, is chemically unstable and susceptible to oxidation. To protect II from oxidation during extraction, freshly prepared ascorbic acid (1.0 *M*) was added to the urine samples prior to extraction. In

the absence of ascorbic acid, the extraction recovery was less than 20%, compared to an average of 85% with ascorbic acid present. Ascorbic acid was also added to injection solutions before overnight HPLC. In addition to the solution instability, II is highly polar and is readily adsorbed onto unmodified silica surfaces. As much as 50% of II at an initial concentration of 10 μg/ml was lost during storage for 24 h in a glass tube and 20% was lost in a glass injection vial during overnight injection. Solutions stored in plastic containers showed no such losses; therefore, standard and injection solutions of II were always stored in plastic containers. The polar properties of II also affected its extraction efficiency and chromatography.

Enzymatic hydrolysis of the conjugates

A recent drug metabolism study [5] showed that significant amounts of II present in urine samples were in the forms of glucuronide and/or sulfate conjugates and could be released by enzymatic hydrolysis. To determine the total (free + conjugated) concentrations of II, urine samples were incubated with 0.5 ml of 'sulfatase', sold by Sigma as a crude solution from Helix pomatia and containing both arylsulfatase and β-glucuronidase, prior to extraction. Under these conditions, the hydrolysis was complete in 1 h at room temperature (Fig. 2). Since the crude enzyme solution contained more than one enzyme, it may be that more than one type of conjugate was hydrolyzed during the incubation. However, no attempts were made to identify the individual conjugates. The hydrolysates were subjected to subsequent extraction and HPLC.

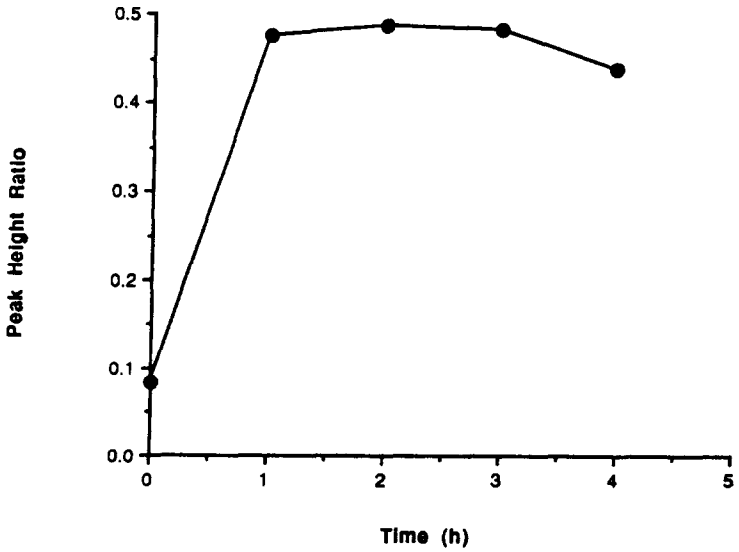

Fig. 2. Effect of incubation time on the enzymatic hydrolysis (crude sulfatase) of II conjugates in 1.0 ml of dosed urine sample.

Extraction

Prior to HPLC, II was isolated and purified from urine samples with solid-phase isolation. Among all reversed phases tested only the phenyl phase was able to retain and extract the highly polar analyte from a urine matrix. The retention was probably due to the affinity between the phenyl moiety of the solid phase and the aromatic moiety of the analyte. For better recovery, II was eluted from the phenyl column with 100% acetonitrile and loaded directly on a silica extraction column without changing to a weaker solvent. The highly polar II was strongly retained on the column and easily separated from most interferences of less polarity during acetonitrile wash. Only aqueous buffer (in this case, citrate buffer) was strong enough to elute the analyte from the silica column. An aliquot of the aqueous eluate was injected directly onto the reversed-phase HPLC column without any further processing. The average extraction recovery was 85%.

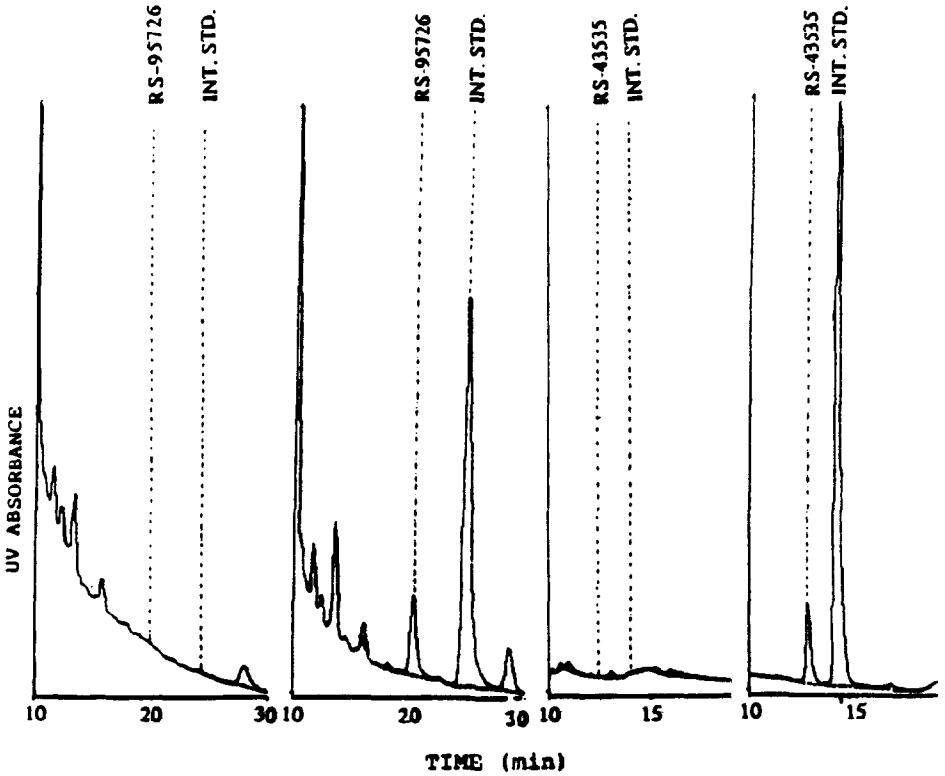

Fig. 3. Representative chromatograms from the analysis of 1.0 ml of control urine spiked with (a) blank solution and (b) 125 ng of II (RS-95726) plus internal standard by means of the method for II, and that spiked with (c) blank solution and (d) 100 ng of III (RS-43535) plus internal standard by means of the method for III.

High-performance liquid chromatography

For final purification and quantification, the extracts were chromatographed on a C_{18} HPLC column. The use of a fully end-capped C_{18} column was essential; otherwise, severe peak broadening and tailing during chromatography was observed for both II and the internal standard, probably due to analyte–silica hydrogen bonding. The use of a base-deactivated silica (BDS) C_{18} column recently developed for basic compounds produced sharper peaks with only minor tailing (Fig. 3b). The use of citrate buffer and tetrabutylammonium phosphate in the mobile phase also helped sharpen the peaks. Addition of THF to the mobile phase provided the required separation between II and the internal standard. Although a relatively weak mobile phase (25% organic) was needed to elute the polar II, no interfering late peaks were found, which indicated an efficient silica-phase extraction. Despite its UV absorption maximum at 260 nm, the absorbance of II was measured at 280 nm, a wavelength selected with the aid of a diode-array detector for optimal specificity and sensitivity. No interferences were found from urine samples, from ascorbic acid added to the injection vials, or from the contaminants contained in the crude solution of sulfatase (Fig. 3a).

Stability, extraction and HPLC of 1,4-diketolonapalene

Compared to II, the method developed for III was simple and straightforward. Since III is an air-stable compound, antioxidant protection during extraction and long-term injection was not needed. Its relatively low polarity resulted in no significant losses of III during storage in glass tubes or injection vials. A one-step C_{18} solid-phase extraction gave better than 90% recovery of III and removed most contaminants in urine. To avoid evaporation, the eluate was diluted with an equal volume of water and was then injected directly onto the reversed-phase HPLC column. Subsequent HPLC with a standard C_{18} column and a simple mobile phase (no THF or tetrabutylammonium phosphate buffer) resolved III and the internal standard. The HPLC system was free from interferences from urine samples and produced sharp and symmetric peaks (Fig. 3c and 3d). III was quantified at 260 nm, which was the absorption maximum.

Linearity, accuracy, reproducibility and limit of determination

The linearity, accuracy, and reproducibility of the method for II was evaluated by quintuplicate determinations. In each determination, urine samples, spiked to give concentrations of II of 125, 250, 500, 1000, or 2000 ng/ml and also spiked with the internal standard, were processed. The peak-height ratio was plotted against the amount of II spiked into each urine sample, and a calibration curve was generated by linear, regression analysis. The validity of the calibration curve was established with a set of urine samples spiked with known amounts of II. The results show that the method is linear over a range of 125–2000 ng/ml with a linear correlation coefficient (r) of 0.9998 or greater. Over the same range, the accuracy as assessed by the error was $<7.2\%$ and the reproducibility as assessed

by the coefficient of variation was <7.1%. Evaluated in a similar manner, the method for III was found to be linear over a range of 100–2000 ng/ml ($r > 0.9997$), accurate (error <2.2%), and reproducible (coefficient of variation <6.9%). If the amount of either metabolite in the dosed sample aliquot was greater than 2000 ng, a smaller aliquot, usually within the range 0.1–1.0 ml, was used for the reanalysis of that sample. The limit of determination at a signal-to-noise ratio of 3:1 was approximately 10 ng for both metabolites.

Application

These methods were used to determine the concentration of II and III in urine samples from a long-term dermal carcinogenicity study of lonapalene. In the study, lonapalene in acetone was applied to the backs of a group of rats at various dosages. Urine samples collected for 20–24 h on designated dates were assayed, total urine volumes were measured, the total mass of each metabolite was calculated for each rat, and the values were averaged for each metabolite determined for a specified dose of lonapalene. Good linear relationships were found between the administered dose of lonapalene and the observed mass of each metabolite for the eighteen-month study (Fig. 4).

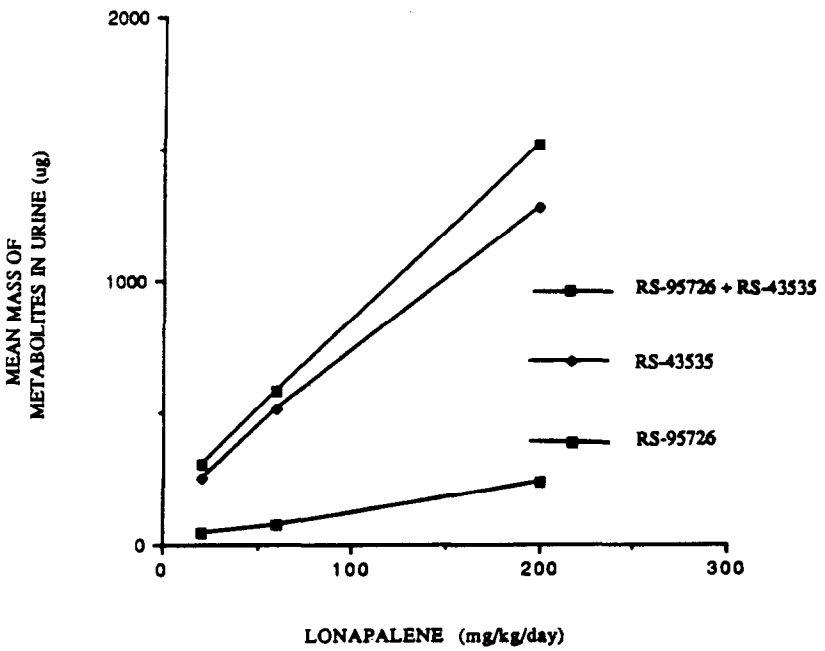

Fig. 4. Relationship between the administered lonapalene dose and the total mean mass of urinary metabolites II (RS-95726) and III (RS-43535) determined after eighteen months of multiple dermal administration of lonapalene to rats.

CONCLUSION

Individual HPLC methods for the determination of II and III in urine samples have been developed. Both methods require no evaporation, and are simple, accurate, reproducible, and suitable for routine analyses. However, the instability and high polarity of II necessitated a great deal of care and attention throughout the procedure. On the other hand, the high polarity allowed a very efficient separation of the analyte from interferents during silica solid-phase extraction. Both methods were employed for the generation of useful metabolic data and are expected to be used for future drug metabolism studies. Use of column switching or backflushing techniques for the determination of both compounds was not attempted. However, with minor modification of the extraction procedure and the mobile phase composition, the method for III could be further developed for simultaneous determination of lonapalene and III.

REFERENCES

1 G. H. Jones, M. C. Venuti, J. M. Young, D. V. K. Murphy, B. E. Loe, R. A. Simpson, A. H. Berks, D. A. Spires, P. J. Maloney, M. Kruseman, S. Rouhafza, K. C. Kappas, C. C. Beard, S. H. Unger and P. S. Cheung, *J. Med. Chem.*, 29 (1986) 1504.

2 A. Lassus and S. Forsstrom, *Br. J. Dermatol.*, 113 (1985) 103.

3 C. T. Jansen, K. Lammintausta, R. E. S. Bullingham and S. Forsstrom, *J. Invest. Dermatol.*, 86 (1986) 137.

4 P. W. Berry, R. V. Tomlinson and J. L. Bowen, *Proc. West. Pharmacol. Soc.*, 30 (1987) 137.

5 M. D. Chaplin and P. W. Berry, Syntex Research, Palo Alto, CA, unpublished results.