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Determination at the Nanogram Range of Rilatinic Acid in Urine After Ion-Pair Extraction

Methylphenidate (α-phenyl-2-piperidineacetic acid methyl ester),[2] synthetized by Panizzon [*1*], is a central nervous system stimulant of the amphetamine series. It differs from many of the phenylisopropylamines in having moderate effects on the peripheral circulatory system. It is used in the management of hyperkinetic and perceptually handicapped children [*2*] where it is claimed to improve both behavior and learning ability [*3*]. It is not only a doping agent for sportsmen but also an increasing factor in the drug abuse problem. In many countries this drug has been submitted to narcotic regulations.

Methylphenidate is extensively metabolized (up to 99.2% in humans) to rilatinic acid (α-phenyl-2-piperidineacetic acid) [*4-6*]. Although a method was described by Schubert [*7*], using flame ionization detection, it is clear that conventional gas-liquid chromatographic (GLC) analysis of the unchanged drug in the urine generally lacks sensitivity. Figure 1 represents the formula of both methylphenidate (*I*) and rilatinic acid (*II*). Derivatization to electron-capturing (EC) compounds such as methylphenidate-*N*-heptafluorobutyrate [*8*] or methylphenidate-*N*-pentafluoropropionate [*9*] greatly improves the detection sensitivity of the unchanged drug.

Although the determination of rilatinic acid (RA) should be the most appropriate parameter to demonstrate methylphenidate ingestion, this analysis is difficult because RA cannot be extracted by organic solvents from aqueous media. Only prior lyophilization of the urine followed by methylation with diazomethane allows the detection of this main metabolite [*6,10*].

The present paper describes the isolation by ion-pair extraction with tetrabutyl-ammonium hydrogen sulfate (TBA) or tetrapentylammonium-hydroxide (TPA) followed by a sensitive thin-layer chromatographic (TLC) detection method. Extractive alkylation with TBA as a counter-ion and pentafluorobenzylbromide as alkylation agent also allows an EC-GLC detection at the nanogram range of the RA-pentafluorobenzyl derivative. This method has been successfully applied to the determination of organic acids [*11-14*].

Materials and Methods

Reagents

Alpha-phenyl-2-piperidineacetic acid (rilatinic acid) was supplied by Ciba Geigy Corporation (Basel, Switzerland). Standard solutions of 10 mg in 50 ml of methanol were prepared.

Tetrabutyl-ammonium hydrogen sulfate (TBA) was obtained from Aldrich-Europe (Beerse, Belgium). A 0.1*M* solution in distilled water was used.

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[2]Trade names: Centedrin®, Rilatine®, Ritalin®, Phenidylate®, and Plimasine® (also contains tripelennamine).

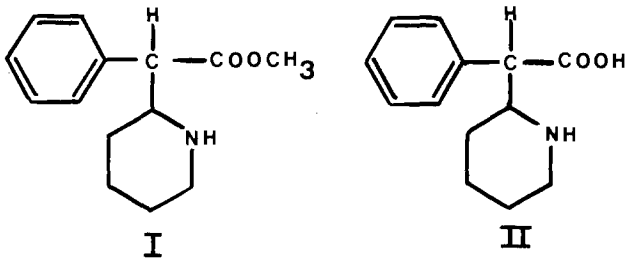

FIG. 1—*Chemical structure of methylphenidate* (I) *and rilatinic acid* (II).

Tetrapentylammonium iodide (TPA-I) was obtained from Fluka AG, Switzerland. Tetrapentylammonium-hydroxide (TPA) was prepared by adding 2.13 g of TPA-I and 0.637 g silver oxide to 30 ml of distilled water, and the mixture was allowed to react for 1 h on a ultrasonic water bath at room temperature. After the precipitate was filtered on a glass filter, the remaining filtrate was extracted twice with 30 ml dichloromethane. The remaining aqueous layer was brought to pH 7.5 with 0.1*M* *o*-phosphoric acid and again extracted with equal volumes of dichloromethane. The TPA remained in the aqueous phase and was used as a 0.1*M* solution.

Pentafluorobenzylbromide (PFB-Br) was obtained from Piers Chemicals (Rockford, Ill.).

Trimethylchlorosilane, 5% in toluene, was used to inactivate the extraction glass tubes.

For the ninhydrin reagent, 0.2 g of ninhydrin was dissolved in 100 ml of acetone.

All solvents for use in the EC-GLC were of high purity.

Pre-coated sheets (Nano-Platten Sil® or Polygram Sil G/UV 254) from Machery-Nagel (Düren, Germany) were used in the TLC analysis.

Apparatus

A Becker gas chromatograph, Model 420, equipped with a 3.7×10^8 Bq (10 mCi) ^{63}Ni pulse-frequency-modulated EC detector was used. The chromatograph was operated with a glass column (2 m by 2 mm inside diameter) inactivated with trimethylchlorosilane and packed with 2.5% OV-225 on Chromosorb W-HP (80–100 mesh). The carrier gas was nitrogen, dried over molecular sieves, at a flow rate of 30 ml/min. At the end of the column an additional 30 ml/min was added to make a total flow of 60 ml/min entering the detector.

An AEI-MS 12 mass spectrometer combined with a gas chromatograph was used under the following operating conditions: accelerating voltage, 8 kV; trap current, 10 μA; and ionization energy, 70 eV. A 1:2 splitter divided the outlet of the column between the chromatograph detector and the mass spectrometer. A membrane separator (Type V 5620, Varian) allowed the eluate to flow into the ion source. The GLC conditions were the same as described above except that helium (60 ml/min) was used instead of nitrogen.

Procedure for the Ion-Pair Extraction

A 1-ml sample of unknown urine is put into a 10-ml glass-stoppered centrifuge tube and the pH is adjusted to 10 with 2*N* sodium hydroxide. One millilitre of TBA (or TPA) solution and 2 ml of dichloromethane are added and the mixture is shaken vigorously on a vortex mixer for 2 min. The layers are separated by centrifuging and the upper layer is aspirated and discarded. The remaining dichloromethane layer is transferred to another glass centrifuge tube and the extract evaporated under a stream of nitrogen. The residue is dissolved in 25 μl of methanol and applied to a TLC plate along with standards of 0.5,

1, and 3 μg of RA. The plate is run first with acetone-acetic acid (98:2) to elute co-extracted impurities and extraneous compounds. The plate is usually run a second time with the same solvent to bring the impurities as much as possible towards the front.

The plate is air-dried and then run with a mixture of methanol/chloroform/acetic acid (50:50:2). After elution the plate is air-dried, sprayed with ninhydrin, and heated at 120°C for 15 min. Violet spots are developed for standards and positive unknowns.

Extractive Alkylation

A 0.5-ml sample of urine is adjusted to pH 10 with 2*N* sodium hydroxide, and 0.5 ml TBA solution, 1 ml dichloromethane, and 15 μl PFB-Br are added. Extractive alkylation is achieved by using a mechanical shaker at room temperature for 30 min.

The two phases are allowed to separate, and the aqueous phase is aspirated and discarded. The organic phase is extracted with 1 ml of 0.5*N* sulfuric acid; the acid layer is washed twice with 1 ml of dichloromethane, alkalinized with solid sodium carbonate, and finally extracted with 2 ml of a mixture of *n*-hexane/ethyl acetate (98:2). Aliquots (2 μl) of this extract are injected into the gas chromatograph at these operating conditions: column temperature, 180°C; injector temperature, 230°C; and EC detector temperature, 300°C. Figure 2 represents the derivatization of RA with PFB-Br.

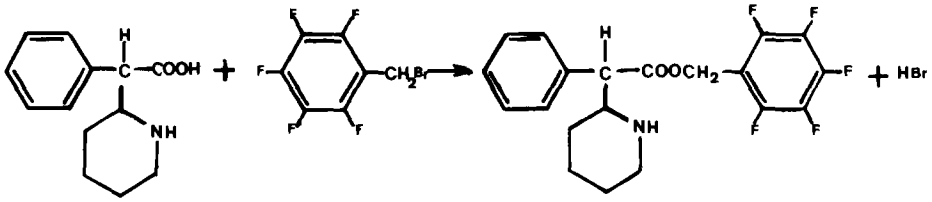

FIG. 2—*Derivatization of rilatinic acid with pentafluorobenzylbromide.*

Results and Discussion

Recoveries for the counter-ion extraction of rilatinic acid from aqueous solutions at pH 10 were previously determined with ultraviolet spectrophotometry. It appeared that a single extraction with dichloromethane using TPA as counter-ion afforded quantitative recovery and that only 70% recovery was obtained when TBA was used, but the extracts obtained with TPA are much more impure than those obtained with TBA because TPA extracted much more extraneous material from the urine. Recoveries from prepared urine samples containing 0.05, 0.2, 0.5, 1, and 2 μg of RA were also evaluated by visually scanning the TLC plates and compared with spots of the same quantities of pure reference standards applied directly on the plate. The recoveries were very satisfactory when TPA was used but were not quantitative with TBA.

Of the several spray reagents tried, ninhydrin quite unexpectedly allowed very sensitive detection of RA (down to 30 ng). This sensitivity allows a rapid and semiquantitative determination of RA in small urine samples. The R_f values and chromatic behavior of rilatinic acid and ephedrine (reference for TLC) are shown in Table 1.

Two consecutive elutions of the plate with an acetone/acetic acid mixture allowed the extraneous materials to move into the upper region of the plate; RA itself remained at the starting point with this solvent.

Figure 3 represents typical gas chromatograms of the pentafluorobenzyl derivative extracted from 1 ml of water to which 1 μg RA had been added (*A*), of an extract prepared

TABLE 1—*The R_f values and chromatic behavior of rilatinic acid and ephedrine.*

Substance	$R_f \times 100$	Chromatic Behavior
Rilatinic acid	32	violet
Ephedrine	37	red-brown

in the same way from a drug-free urine (*B*) and of a positive urine, collected 4 h after oral ingestion of a single (10 mg) therapeutic dose of methylphenidate (*C*).

Although the EC examination allowed the detection of RA concentrations as low as 1 ng, this method cannot be used for quantitative determinations of RA, but valuable confirmation of RA in suspected urines is possible. Indeed, the derivatization yields not a single product (the expected mono-pentafluorobenzyl derivate, as shown in Fig. 2) but rather a substantial quantity of another compound, probably derivated on both the carboxyl and NH functions. Furthermore, quantitative derivatization on a micro scale could not be realized with the actual operational conditions. The peaks at 3.3 and 4.1 min are always present in normal urine samples and have not yet been identified. The identity of the peak at 5.3 min was confirmed by gas chromatography-mass spectrometry under the conditions described above. The mass spectrum is presented in Fig. 4 and corresponds perfectly with the expected structure. The dipentafluorobenzyl derivative of RA elutes from the column only after 34 min.

Summary

Ion-pair extraction and extractive alkylation of urine allows a simple, rapid, and very sensitive detection by TLC as well as EC-GLC techniques, of rilatinic acid in small urine samples after the oral intake of therapeutic doses of methylphenidate.

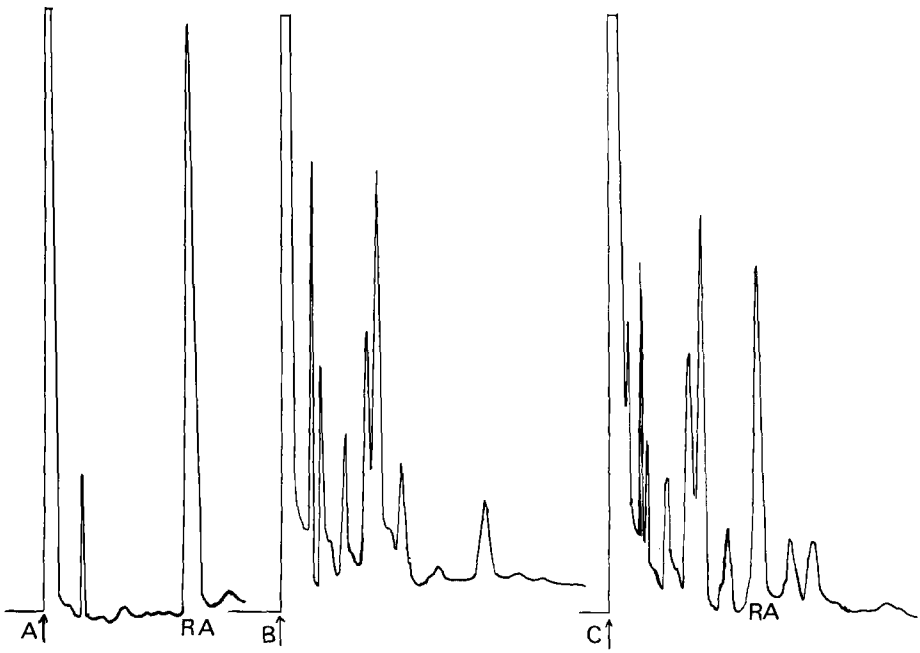

FIG. 3—*Typical gas chromatograms obtained from* (A) *aqueous rilatinic acid solution,* (B) *drug-free urine, and* (C) *a positive urine.*

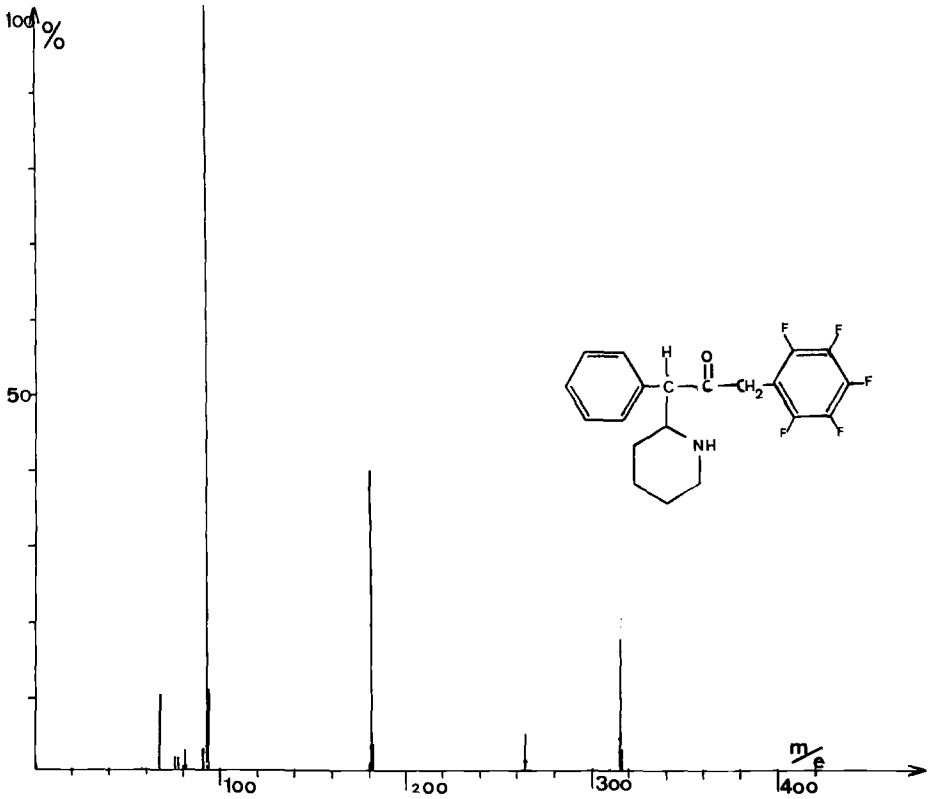

FIG. 4—*Mass spectrum of the rilatinic acid-pentafluorobenzyl derivative.*

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