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Simultaneous identification of amphetamine and its derivatives in urine using HPLC-UV

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Summary. An HPLC-UV method for the simultaneous identification of amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylenedioxymethamphetamine (MDMA) in urine is described. It includes a rapid extraction procedure of the 4 analogs from urine using Extrelut 3 columns, derivatization with sodium 1,2-naphthoquinone-4-sulphonate (NQS) to obtain highly chromophoric UV-VIS derivatives, and a final HPLC analysis using an ion-pair reversed-phase technique with eluent monitoring at 480 nm. Structural characterization of the derivatives obtained by mass spectrometry is reported. Recoveries of the amphetamines were in the range 80–85% at concentrations of 300 ng/ml. Practical detection limits were 40–60 ng/ml (S/N ratio = 10) for all derivatives. The chromatographic peaks of the NQS derivatized amphetamines are fairly narrow and well resolved. The method is simple, rapid, quite sensitive, and specific for convenient confirmation of preliminary positive results obtained with immunoassays.

Key words: Amphetamines – Reversed-phase liquid chromatography – UV detection

Zusammenfassung. Eine HPLC-UV-Methode zur gleichzeitigen Bestimmung von Amphetamin, Methamphetamin, 3,4-Methylendioxyamphetamin (MDA) und 3,4-Methylendioxymethamphetamin (MDMA) im Urin wird beschrieben. Vorgestellt wird eine Schnellextraktion der 4 Amphetamine über Extrelut-3-Säulen und eine Derivatisierung mit Natrium-1,2-naphthochinon-4-sulfonat (NQS) um hochchromophore UV-VIS-Derivate zu erhalten. Abschließend erfolgt eine HPLC-Analyse mittels einer reversed phase Technik (Ionenpaar) mit Detektion des Eluats bei 480 nm. Die Struktur der NQS-Derivate wird durch Massenspektrometrie charakterisiert. Die Wiederfindungsraten der Amphetamine liegen bei Konzentrationen von 300 ng/ml bei 80–85%. Die Nachweisgrenze liegt für alle Derivate bei 40–60 ng/ml (S/N-Verhältnis = 10). Die Trennung der NQS-derivatisierten Amphetamine gelingt gut, die Methode ist einfach, schnell, empfindlich und spezifisch genug für die Bestätigung vorläufiger Screening-Resultate, wie sie mit Hilfe von Immunoassays erhalten werden.

Schlüsselwörter: Amphetamine – Reversed-phase-Flüssigkeitschromatographie – UV-Detektion

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Introduction

During the past two decades the demand for psychoactive substances has led to the growing problem of illicit clandestine laboratories. The manufacture, trafficking and abuse of clandestinely produced drugs is a worldwide problem, and international trafficking in precursors, chemicals, solvents, reagents and catalysts often proceeds under the guise of legitimate commerce. Within the wide variety of clandestinely produced synthetic substances, designer drugs and analogs, narcotics, depressants, stimulants and hallucinogenics, the widespread abuse of amphetamine analogs has markedly increased, becoming a serious social problem [1].

To attain aims of clinical and forensic relevance, toxicology laboratories are involved in developing reliable analytical procedures to detect the presence of amphetamine (AMP), methamphetamine (MEAMP), 3,4-methylenedioxyamphetamine (MDA), and 3,4-methylenedioxymethamphetamine (MDMA) in biological fluids. Among techniques suitable for confirming urine immunoassays, high pressure liquid chromatography (HPLC) [2] is a valid alternative technique to gas chromatography/mass spectrometry (GC/MS) [3–6]. Although very sensitive and specific, some HPLC methods are still impractical for the confirmation of amphetamines in urine, because of lengthy extraction steps or not easily available or usable fluorescent and chemiluminescent detectors [7–9].

This paper reports an HPLC-UV method for the simultaneous identification of AMP, MEAMP, MDA and MDMA in urine, including a rapid extraction procedure, derivatization to obtain highly chromophoric UV-VIS derivatives, and analysis by the ion-pair reversed-phase technique.

Materials and methods

Materials. Methanol, n-hexane and carbon tetrachloride of analytical grade, and acetonitrile and water of HPLC grade, were obtained from Merck (Darmstadt, Germany).

Potassium hydroxide, hydrochloric acid, sodium bicarbonate, potassium hydrogen phosphate, methanesulphonic acid and sodium 1,2-naphthoquinone-4-sulphonate (NQS) were of analytical grade and purchased from Merck. Extrelut 3 columns were obtained from Merck. AMP, MEAMP, MDA and MDMA (Division of Narcotic Drugs, United Nations, Vienna, Austria) were dissolved in methanol to prepare stock solutions at a concentration of 1.5 mg/ml. To test the extraction procedure, 2 levels of these drugs (0.5 and 5 µg/ml) were prepared by adding appropriate amounts of the stock solutions to urine samples from healthy volunteers. Appropriate dilutions of the standard solutions with the HPLC mobile phase provided working standards.

Urine samples, obtained from local addiction medicine units, were preliminarily screened as positive for amphetamines by EMIT d.a.u. monoclonal assay.

Extraction procedure. A 3-ml aliquot of urine was adjusted to pH 11 with 10 N potassium hydroxide and poured into an Extrelut 3 column. One drop of 3 N hydrochloric acid was poured inside the collection tube and after 10 min, elution was carried out with 15 ml of n-hexane. The eluate was evaporated to dryness under a gentle stream of nitrogen at 35°C, 1.5 ml 8% aqueous solution of sodium bicarbonate and 1 ml 0.5% aqueous solution of NQS were added to the residue. The resulting mixture was heated at 70°C for 60 min. After cooling, it was extracted with 5 ml of carbon tetrachloride, and the resulting organic phase was evaporated to dryness under a gentle stream of nitrogen at 40°C. The residue was reconstituted with 100 µl of HPLC mobile phase and a 20-µl aliquot was injected into the chromatograph.

Apparatus and chromatographic conditions. The HPLC apparatus consisted of: Merck Hitachi L 6200 intelligent pump; L 4200 UV-VIS detector; D 2000 Chromato-integrator; Rheodyne 7125 injection valve with a 20 µl sample loop (Rheodyne Corp., Berkeley, CA). A 250 × 4 mm Hibar analytical column (Merck) packed with Lichrospher 100 RP18 (5 µm) and a Lichrocart 4-4 pre-column (Merck) packed with Lichrospher 100 RP18 (5 µm) were used. The eluent was monitored at 480 nm. Chromatographic separation was accomplished at room temperature using the following mobile phase: phosphate buffer (pH 3; 0.03 *M*) containing 0.05 *M* methanesulphonic acid : acetonitrile (48 : 52, v/v). Flow rate: 0.65 ml/min. The phosphate buffer was prepared by dissolving 4.083 g of potassium dihydrogen phosphate in 950 ml of water; 3.25 ml of methanesulphonic acid were added and adjusted to pH 3 with 5 N potassium hydroxide. The resulting mixture was diluted with water to a final volume of 1000 ml.

All liquid chromatographic solvents were filtered before use through ANODISC 47 (0.2 µm) filters (Merck) and degassed under vacuum with stirring.

Mass measurements. All mass spectrometric measurements were performed on a VG-ZAB-2F instrument [10] operating in EI conditions (70 eV, 200 µA, 200°C source temperature). Samples were introduced *via* a direct inlet system with a probe temperature of 280°C. Metastable transitions were detected by mass-analysed ion kinetic energy (MIKE) spectroscopy [11].

Results and discussion

The derivatization of sympathomimetic amines with NQS was first applied by Gürkan [12] and Hashimoto et al. [13]. Endo et al. [14] described a method for the determination of AMP and MEAMP in urine by NQS derivatization and straight-phase HPLC analysis. Farrell and Jefferies [15] reproduced the results reported by Endo et al. [14] and also compared other HPLC methods for amphetamines including several ion-pair reversed-phase techniques.

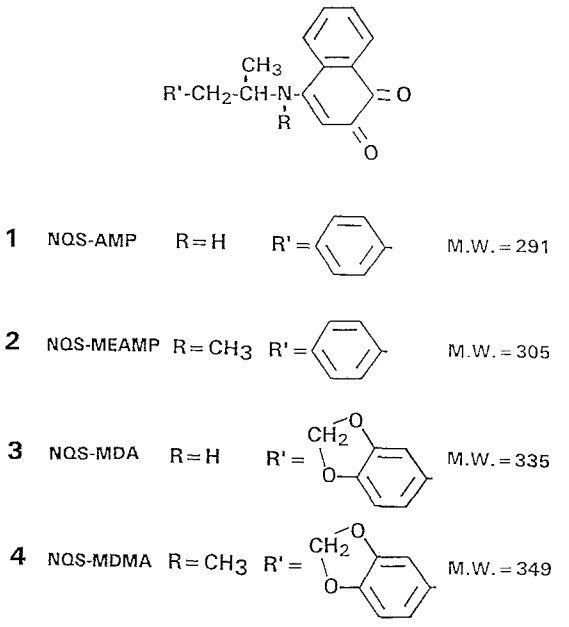

Fig. 1. Chemical structures of NQS derivatives of AMP, MEAMP, MDA and MDMA

For the first time, to the best of our knowledge, this paper reports the derivatization of MDA and MDMA with NQS to obtain the corresponding chromophoric derivatives. These compounds were separated, together with the AMP and MEAMP derivatives, using a reversed-phase ion-pair HPLC technique. With respect to the above studies, the extraction of the amphetamine analogs from urine and of the derivatives from the reaction medium was slightly modified. Furthermore, the HPLC analysis was optimized using methanesulphonic acid as ion-pairing reagent.

We found that the Extrelut 3 columns represented an effective alternative to the conventional liquid-liquid extractions, avoiding incidental emulsions and improving the speed of sample preparation. The use of n-hexane permitted efficient extraction of the 4 sympathomimetic amines from urine, reducing the co-extraction of polar urinary components. Lower recoveries and less clean extracts were obtained using the following solvents or mixtures of solvents: diethyl ether, ethyl acetate, chlorobutane, dichloromethane : isopropyl alcohol (85 : 15), n-hexane : chlorobutane (50 : 50), n-hexane : diethyl ether (75 : 25).

The coloured NQS derivatives of the 4 amphetamine analogs (Fig. 1) were obtained as reported by Endo et al. for AMP and MEAMP [14], using a slightly larger volume of the 8% sodium bicarbonate solution and a longer reaction time (60 min), in order to obtain good reaction yields in a reasonable time for all derivatives.

Structural characterization of the derivatives was required as a first step in developing the complete HPLC

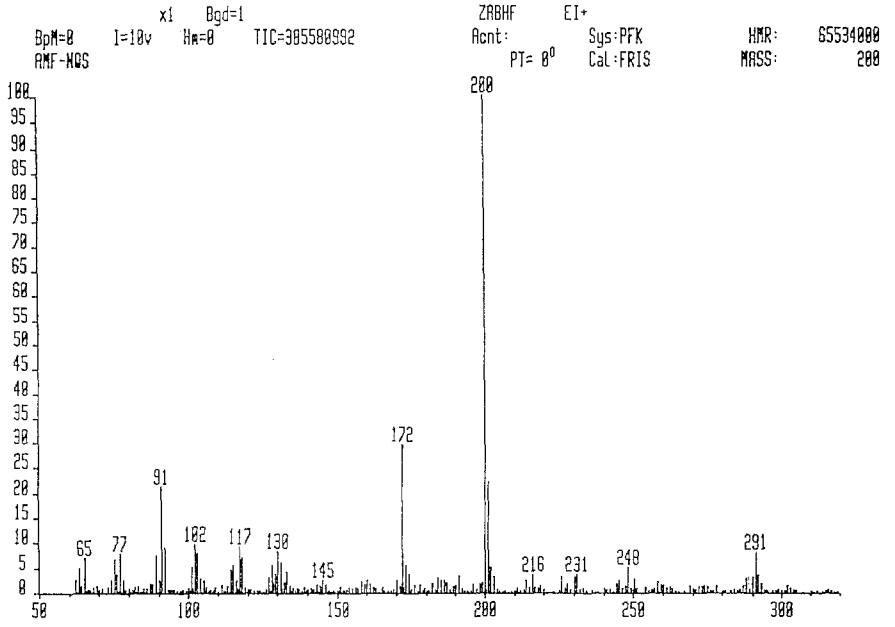

Fig. 2. EI mass spectrum of NQS derivative of AMP

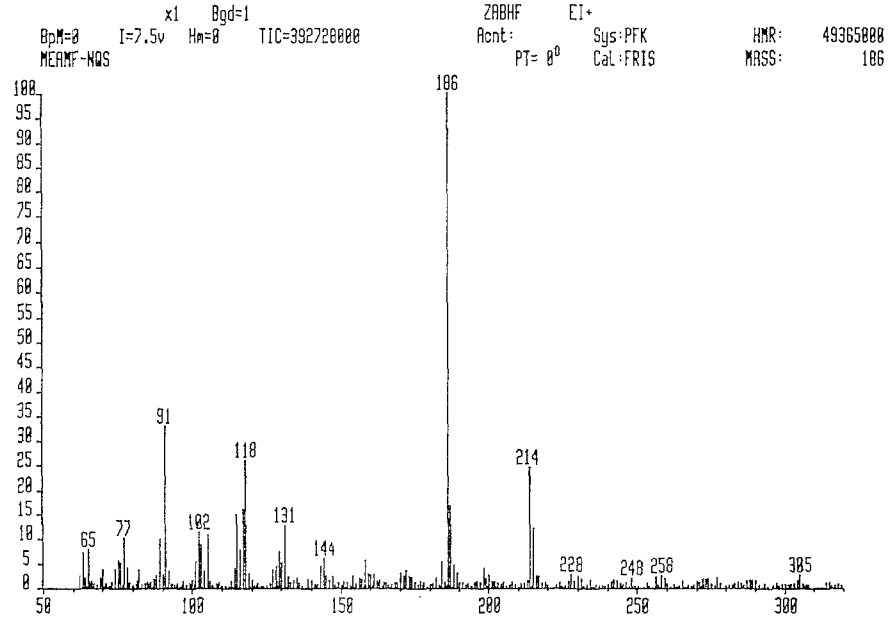

Fig. 3. EI mass spectrum of NQS derivative of MEAMP

method, concerning in particular the new derivatives of MDA and MDMA. Such structural characterization was obtained by synthesizing every derivative and analysing the resulting samples by EI mass spectrometry and MIKE spectroscopy [11]. Figs. 2–5 show the EI mass spectra of the 4 derivatives. Even though the molecular ions detected for all compounds were not in abundance their EI-induced decompositions were highly diagnostic from the structural point of view. This allowed confirmation of previous reports for AMP and MEAMP [13] and of the expected structures for the new derivatives of MDA and MDMA. All derivatives were found to be stable for at least 10 days. Extraction of the derivatives from the cooled reaction mixtures was optimized in terms of efficiency and cleanliness by using carbon tetrachloride, which gave better results than chloroform, n-hexane, n-hexane: ethyl acetate (85:15), n-hexane: diethyl ether (75:25), toluene. Carbon tetrachloride, however, needs to be handled with caution, since its hepatotoxicity (centrilobular necrosis, accumulation of triglycerides) and carcinogenicity have been documented [16].

A reversed-phase ion-pair HPLC technique was employed for the analysis of the 4 NQS derivatives. Methane sulphonic acid gave the best chromatographic performance with respect to ion-pairing reagents such as hexane sulphonic acid or heptane sulphonic acid.

Although the derivatives showed stronger absorption bands in the UV range than in the visual range, detection

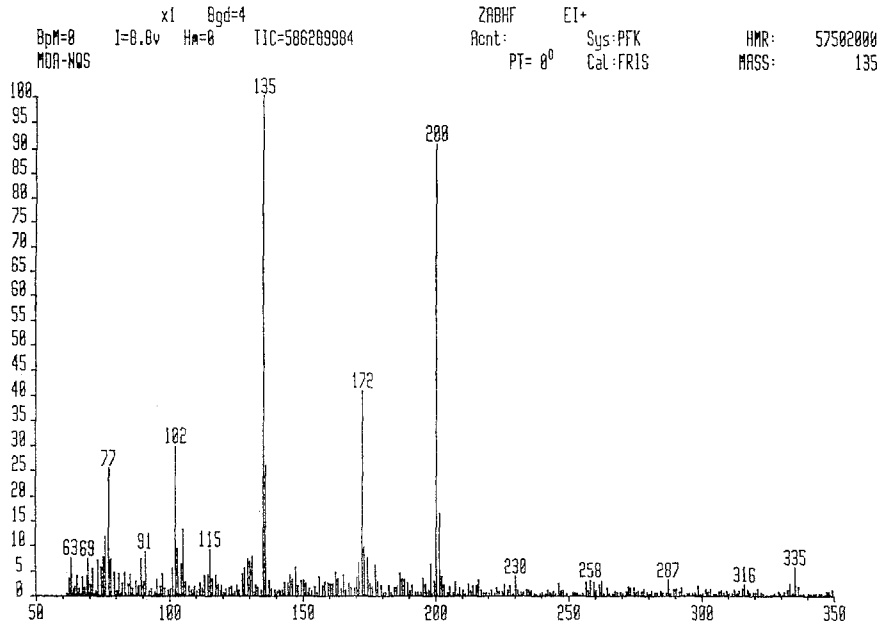

Fig. 4. EI mass spectrum of NQS derivative of MDA

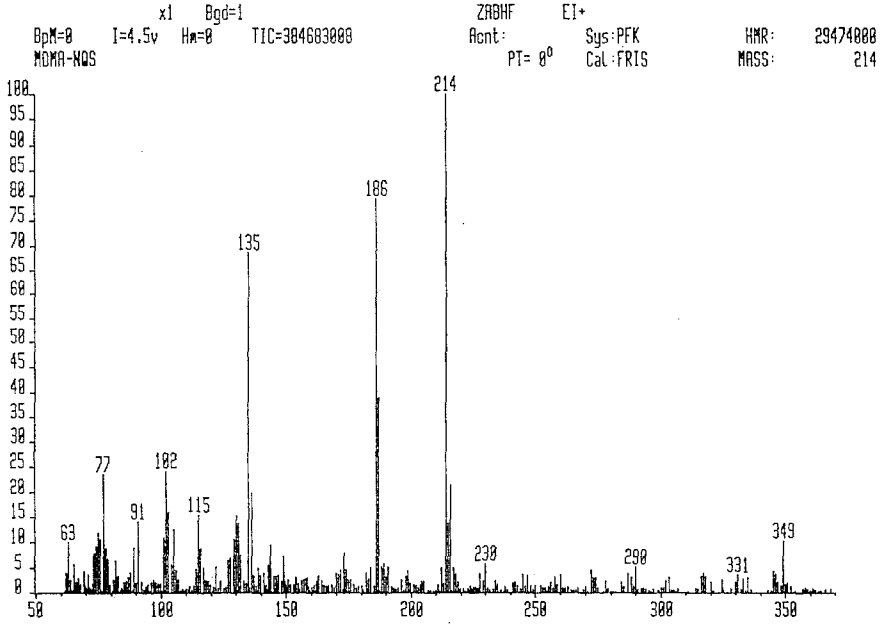

Fig. 5. EI mass spectrum of NQS derivative of MDMA

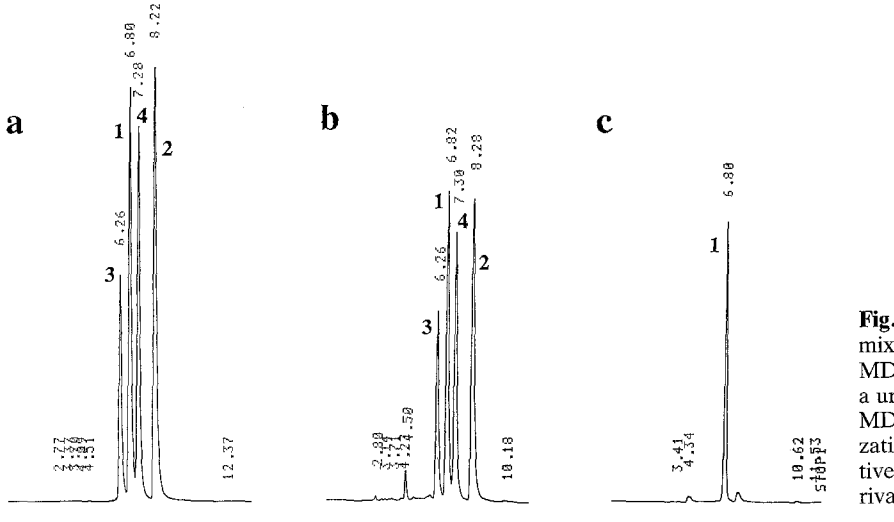

Fig. 6a–c. Liquid chromatograms of a standard mixture of AMP (1), MEAMP (2), MDA (3) and MDMA (4) derivatized with NQS **a**; an extract of a urine sample spiked with AMP, MEAMP, MDA and MDMA (1 µg/ml each) after derivatization with NQS **b**; an extract of an EMIT positive urine sample for amphetamines (after derivatization with NQS) **c**

Table 1. Basic drugs found not to interfere with HPLC procedure

Amitriptyline	Nicotine
Caffeine	Orphenadrine
Carbamazepine	Phendimetrazine
Chloroquine	Phenylpropanolamine
Chlorpromazine	Phenmetrazine
Desipramine	Phentermine
Doxapin	Procaine
Ephedrine	Procainamide
Fenfluramine	Prometazine
Imipramine	Pseudoephedrine
Lidocaine	Ranitidine
Loxapine	Theobromine
Meperidine	Theophylline
Mephentermine	Trimipramine
Methylenedioxyethylamphetamine	

was preferably carried out at 480 nm, a highly characteristic wavelength for all derivatives, for better specificity.

Positive identification of the NQS-derivatized amphetamines was based on elution times, absorbance maxima and comparison with standards.

Recoveries of AMP, MEAMP, MDA and MDMA from urine, employing the described method, were in the range 80–85% at concentrations of 300 ng/ml. Practical detection limits, with a signal-to-noise ratio of 10:1, were in the range 40–60 ng/ml for all analytes.

The sensitivity and specificity of the method appear to be suitable for the confirmation of preliminary positive results obtained by EMIT assay, since they allow the detection of urinary levels of amphetamines below the cut-off level of the EMIT assay, as well as clear distinctions among the 4 analogs. Total analysis time, including extraction and the HPLC run is about 2 h. Typical chromatograms obtained by HPLC analysis of the above drugs are shown in Fig. 6. As may be seen, the peaks of the 4 derivatives are fairly narrow and well resolved. The absence of the solvent front, as well as the good baseline stability, represent indirect practical advantages related to the chosen working wavelength of 480 nm.

The basic drugs listed in Table 1 were found not to interfere with the HPLC determination of the 4 amphetamine analogs. Some of them do not react or form stable derivatives with NQS, e.g. phenylethylamine derivatives containing a hydroxyl group [13]. Others gave chromatographic peaks which were clearly separated from the four studied here.

In conclusion, this study indicates that the reported method is simple, rapid, quite sensitive and specific. It is suitable for routine detection of the presence of AMP, MEAMP, MDA and MDMA in urine samples and can be conveniently applied to confirm preliminary positive results obtained by immunoassays.

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