Journal of Chromatography, 143 (1977) 203—206
Biomedical Applications

CHROMBIO. 017

Note

Rapid analysis of nicotine and cotinine in the urine of smokers by isocratic high-performance liquid chromatography

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(Received April 14th, 1976)

Nicotine and cotinine can be detected using ultra-violet spectroscopy after basic extraction [1], and may be separated by thin-layer chromatography [2]. There are several methods utilising gas chromatography (GC) in use [3—5]. Thin-layer chromatography is a good qualitative method, ultra-violet spectroscopy will not differentiate between nicotine and cotinine, GC, although rapid and sensitive, does not allow rapid analysis of nicotine and cotinine, temperature programming being required, or replicate analysis of samples at a higher temperature [3].

Distillation [6] and multiple extraction [3] have been used to obtain complete recovery prior to estimation. Using the modification of Bell and Stewart [7] of the extraction procedure for amphetamine estimation [8], and an appropriate internal standard, a rapid, quantitative method for estimation of urinary nicotine and cotinine was evolved using high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Nicotine was obtained from BDH (Poole, Great Britain) and a standard of 61.7 μmole/l in 0.1 *M* hydrochloric acid was prepared. Quinoline was also obtained from BDH, desmethylimipramine was donated by Mr. R. Sparks (Department of Pharmacology, Ninewells Hospital and Medical School, Dundee, Great Britain). Cotinine was prepared by the Dundee City Analyst. The bromothymol blue and the sodium hydroxide were also obtained from BDH.

CHROMATOGRAPHY CONDITIONS

A Varian 8500 chromatograph, with a Varian 635 series spectrophotometer was used. The stainless-steel column (25 cm × 2 mm I.D.) was packed with Micropak SI-10 silica gel. The operating conditions were as follows: temperature, ambient; pressure, 550 p.s.i.; flow-rate, 40 ml/h; chart speed, 25 cm/h; slit width, 1 nm; wavelength, 260 nm; solvent, ethyl acetate—propan-2-ol—ammonia (80:3:0.4).

EXTRACTION PROCEDURE

A 3-ml volume of urine in a conical centrifuge tube was made alkaline to 1% bromothymol blue using 5 *M* NaOH. 0.3 ml of the desmethylimipramine or quinoline internal standard was added and the sample was mixed for 30 sec on a whirlimixer. The tubes were centrifuged to clear any emulsions formed and 10 μl injected onto the column.

RESULTS

Recovery

Recoveries of 98—104% relative to the internal standard were obtained on standard samples.

Standards

A calibration curve for nicotine showed linearity over the range 0.6—60 μmole/l.

Desmethylimipramine was the internal standard for HPLC work. Quinoline was the internal standard for the GC method used to confirm the results obtained by HPLC.

10 μg/ml of desmethylimipramine and quinoline were dissolved in dichloromethane. This is equivalent to a concentration of 77.5 μmole/l of desmethylimipramine and 37.5 μmole/l of quinoline. As the available cotinine was not pure, cotinine peaks were calculated as nicotine.

Sensitivity

By suitable dilution of a standard solution the minimum detectable level was found to be 5 ng on column, i.e. equivalent to a concentration of 300 nmole/l.

Separation

Fig. 1 is a trace obtained from an extract of a smoker's urine, showing nicotine and cotinine with the internal standard desmethylimipramine. Fig. 2 shows the trace obtained when an extract from a non-smoker is chromatographed.

Precision

Replicate analysis of a smoker's urine showed a coefficient of variation of 7.3% for nicotine, 9.0% for cotinine, and the coefficient of variation of the calculated nicotine:cotinine ratios was 11.1%.

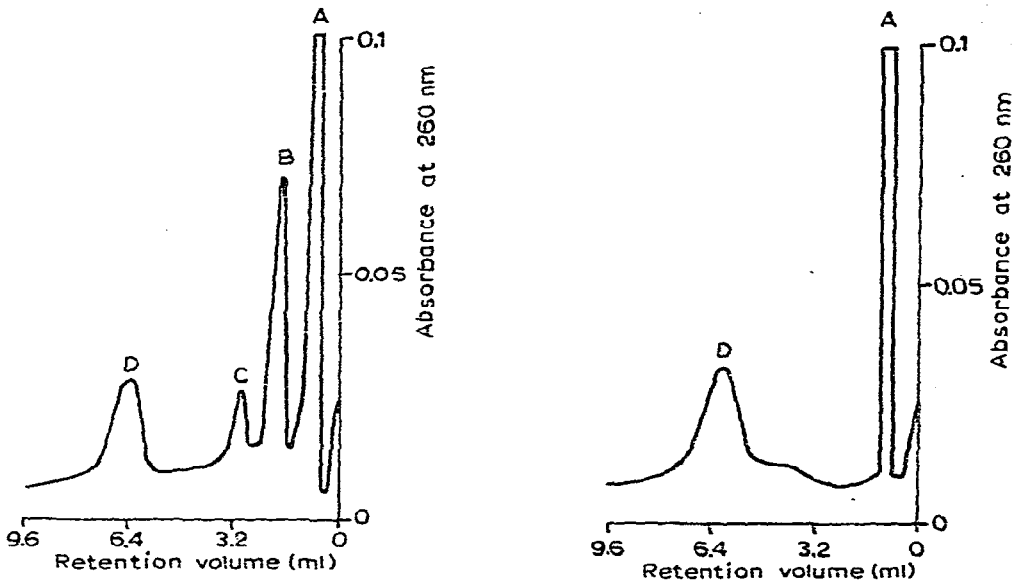

Fig. 1. Chromatogram of a smoker's urine. A = Solvent front, B = nicotine, C = cotinine, D = desmethylimipramine. Absorbance range, 0—0.1.

Fig. 2. Chromatogram of a non-smoker's urine. A = Solvent front, D = desmethylimipramine. Absorbance range, 0—0.1.

Specificity

As a tricyclic antidepressant is used as the internal standard, it is to be expected that such drugs might interfere with the analysis. No interference was noted from the common tricyclic antidepressants. Interference when found in HPLC, did not occur in the GC method. Similarly interference in the GC method was not observed in the HPLC method.

Correlation

Nicotine levels using the HPLC method were correlated with the GC method of Bell and Stewart [7]. Using 38 specimens, a correlation coefficient of 0.986 was found.

DISCUSSION

The major advantage of HPLC over the GC methods available, is the rapidity of elution of nicotine and cotinine (within 5 min), although if precise results are required a further 5 min are needed for elution of the HPLC internal standard. Work up time of specimens is fast, being based on the method of Ramsay and Campbell [8] for amphetamine. Previous published methods for nicotine and cotinine have involved lengthy extraction procedures [3, 6].

Solvent concentrations were found to be critical for peak shape and retention times, the latter is particularly sensitive to the degree of deactivation caused by propan-2-ol.

It has been reported that nicotine levels of 100—3000 μg/l (617 nmole/l—18.5 μmole/l) have been found in smokers' urine [9]. The results obtained in this investigation agree with these levels, nicotine concentrations ranging from 300 nmole/l—18.3 μmole/l, cotinine levels ranging from 250 nmole/l—3.45 μmole/l, and nicotine:cotinine ratios varying from 0.54—22.9. Most variation in the ratios appears to be attributable to the wider range of nicotine levels found. Cotinine is less frequently detected than nicotine.

The same authors claim that levels of 150 nmole/l of nicotine are seen in the urine of non-smokers, rising to 1.54 μmole/l when exposed to a smoky atmosphere. It is doubtful if the method presented here would have sufficient sensitivity to detect the lower limit precisely.

The chief advantage of this method is its rapidity in the estimation of nicotine and cotinine. Simultaneous nicotine and cotinine estimations could be performed by GC, but require temperature programming and are time consuming.

The use of HPLC eliminates the need for lengthy analysis times. Thus specimens from an anti-smoking clinic can be analysed as a patient waits, and objective evidence can be obtained of the truth of their statements that they have stopped or cut down, based on the nicotine levels measured. Nicotine metabolism may relate to the amounts detected.

The estimation of cotinine simultaneously with nicotine might be of use in metabolic studies in smokers.

ACKNOWLEDGEMENTS

I would like to thank Dr. M.J. Stewart for supplying the samples and for checking the manuscript.

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Journal of Chromatography, 143 (1977) 207—209
Biomedical Applications

CHROMBIO. 015

Note

Probenecid, a possible interferent in the gas chromatographic determination of diphenylhydantoin

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(Received April 2nd, 1976)

The on-column alkylation of barbiturates and related compounds with reagents such as tetramethylammonium hydroxide (TMAH) or triethylanilinium hydroxide (TEAH) is a frequently used technique for the quantitative analysis of these compounds [1—6]. As these determinations are usually performed on sera from patients suffering from epilepsy with the object of helping the clinician to establish the optimal dose regimen, it is imperative that one should be aware of any drugs that may influence the quantitation of the antiepileptic drugs or produce a false positive result. We have found probenecid to be such a drug.

METHOD FOR THE ANALYSIS OF BARBITURATES AND DIPHENYLHYDANTOIN

Our laboratory is engaged in the routine analysis of phenobarbitone and diphenylhydantoin for an epilepsy clinic and we make use of the following method. To 1 ml serum are added secobarbitone and tolylphenylhydantoin as internal standards. The serum is acidified with 0.5 ml of 1 *M* H_3PO_4 and extracted with 3 ml of toluene by shaking for 2 min on a Whirlimixer. After centrifuging to separate the layers, the toluene phase is transferred into a conical centrifuge tube and 100 µl of 0.5 *M* TEAH solution in ethanol and 20 µl of water are added. After vigorous shaking for 2 min on a Whirlimixer, the tube is centrifuged and 4 µl of the bottom layer are injected during 7 sec into the gas chromatograph.

The ratios of the peak areas of N-ethylphenobarbitone and N-ethyldiphenylhydantoin to those of the two internal standards are used to quantitate the drugs.