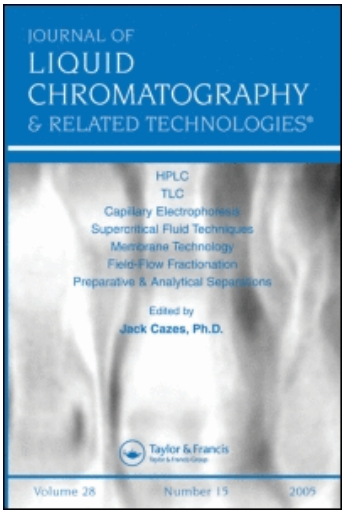

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REVERSED PHASE HPLC AS A METHOD OF REVEALING NITROMETHANE-INDUCED FALSE HYPERCREATININEMIA

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ABSTRACT

A reversed phase HPLC method was developed to quantify rat serum creatinine and overcome the lack of specificity of the colorimetric Jaffé reaction.

C_8, C_{18} and phenyl phases were prepared for comparison. The phenyl phase offers the best compromise between a short analysis time and a good selectivity of separation with isocratic elution.

The sera of rats exposed to nitromethane vapors or treated with $HgCl_2$ exhibited an increase in the Jaffé reaction as compared with controls. HPLC revealed hypercreatininemia in the sera of $HgCl_2$-treated rats but not in those of nitromethane-exposed rats. These results support the conclusion that the overestimated reaction due to nitromethane was a false positive response. Similar analytical interference was also found in sera loaded with nitromethane, but not with nitroethane or 2-nitropropane.

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INTRODUCTION

Various false responses have been shown to occur in clinical laboratory test procedures with medications and several critical reviews deal with this problem (1, 2). Though laboratory test procedures are commonly used to detect health-related effects manifested in humans and experimental animals exposed to occupational pollutants, little work has been undertaken to identify industrial chemicals which may alter the laboratory test values (3).

In this work, we report in vivo investigations demonstrating that the sera of rats exposed to nitromethane (NM) develop an overestimated Jaffé reaction, with no sign of nephrotoxicity. Consequently, a high-performance liquid chromatography (HPLC) method derived from those recommended to improve the specificity of creatinine determination in humans (4, 5, 6, 7, 8) was carried out to examine its appropriateness as a means of avoiding NM-induced interference. Both methods were then compared in the detection of true hypercreatininemia due to $HgCl_2$ (9).

Further, in vitro studies were carried out to account for NM-induced interference with creatinine content and to determine whether two other nitroalkanes, nitroethane (NE) and 2-nitropropane (NP) could affect the Jaffé reaction.

MATERIALS AND METHODS

Reagents and Solvents

Octyltrichlorosilane, octadecyltrichlorosilane, phenyltrichlorosilane and trimethylchlorosilane were purchased from Merck, Darmstadt,

West-Germany and were used without further purification. All other products (Merck) used in experimentation and synthesis were respectively of reagent and chromatographic grades. Synthesis solvents were dehydrated on molecular sieves. Water was produced with a Milli-Q reagent-grade water system (Millipore Corp., Bedford). Lichrosorb Si 60 5 μ was used in the preparation of bonded phases. Commercial kits and quality control sera used for the colorimetric determination of creatinine were purchased from Boehringer Mannheim, France.

Equipment

HPLC analysis was carried out with a Waters Assoc. Model 590 chromatography pump in conjunction with a 10 μl loop sample injector (Rheodyne model 7125) and a LC-UV variable wavelength absorbance detector (Pye Unicam PU 4020). An integrator (Spectra Physics SP 4020) or a recorder (Sefram) was used for quantification of the results. Colorimetric determinations were performed on a Cobas-Bio Analyser (Roche Instruments)

HPLC Support Preparation

C_8, C_{18} and phenyl phases were synthesized and reaction conditions were controlled to produce a low and a high percentage of derivatization. Weakly covered reversed phases were performed according to Evans (10) and highly covered ones by the same method, but with increased reaction times and trichlorosilane concentrations. Anhydrous silanisation reactions were effected at room temperature (10). Silicagel (2.5 g) was reacted in carbon tetrachloride (50 ml) with either trichlorosilanes $0.75.10^{-3}$ M for 2h or $3.65.10^{-3}$ M for 12h. The

bonded phase was washed consecutively with dry CCl_4, to remove unreacted silane, dry methanol and dry methylene chloride. After suction drying, the prepared material was "end capped" with an excess of trimethylchlorosilane in the conditions previously described. The carbon content of each stationary phase was determined in duplicate by microanalysis ; no pair of duplicates differed more than 0.3%. Carbon analyses were performed at the CNRS (Vernaison, France).

HPLC Column Preparation

The 20 cm x 4.5 mm i.d. columns were prepared by the balanced density slurry packing method using toluene, 2-propanol and 95% ethanol (1/1/1). Pressure was set at approximately 40,000 KPa.

HPLC Creatinine Determination

Precipitation of proteins was obtained by adding 50 µl of trichloroacetic acid (10% w/v) to 200 µl of serum. The mixture was vortex mixed for 30 sec., allowed to stand at room temperature and then centrifuged at 9980 g for 3 min. 10 µl of the supernatant were directly injected into the column. The chromatographic elution was done isocratically with $3. 10^{-2}$ M aqueous ammonium acetate at a flow rate of 1 ml/min. The UV detector was set at 285 nm and 0.02 AUFS for sensitivity. The reversed phenyl phase column was chosen among other columns performed. Quantification was achieved by external calibration. The sensitivity of detection at a peak/noise ratio of 5/2 was 0.3 mg/l creatinine.

Colorimetric Creatinine Determination

The Jaffé reaction of creatinine with picrate in alkaline solution to form a reddish-brown 1:1 adduct was used (11, 12).

Based on a kinetic method, the two-point reaction rate of color development was performed without deproteinization of the serum and monitored at 490 nm within 2 min. A standard solution of creatinine (20 mg/l) was employed for calibration. Qualitity controls were systematically performed using Precinorm S specimens.

Experimental Design

In the "in vivo" study, four test groups of 5 rats were exposed in inhalation chambers to 495, 926, 1502 and 1738 ppm of NM for a 4h period. The control groups were exposed to clean filtered air. Each exposure level was checked by gas-liquid chromatography at 70°C, in a column (2 meters long) packed with diethylene glycol succinate, using acetonitrile as an internal standard. Three other groups were subcutaneously injected with 2,4 and 5 mg/kg body weight of $HgCl_2$ dissolved in a saline solution (0.9 % w/v). A control group received only saline solution. Blood samples were taken 24h after the end of each treatment.

In the "in vitro" study, pools of sera were supplemented with NM, NE or NP in order to obtain a wide range of concentrations of each test chemical in one pool.

Differences between control and exposed groups were analyzed statistically by the Mann Whitney test. The level of significance was set for $P < 0.05$.

RESULTS

Chromatographic Conditions

Prepared columns were tested to quantify the serum creatinine and to determine its k'. The influence of the carbon content and

the nature of the bonded-phase sorbent was observed at different concentrations of ammonium acetate (table 1). With the exception of the phenyl phase, whatever the salt concentration of eluant, the shortest k' were always found with high percentages of carbon-loading C_8 and C_{18} silicagels, as compared with weak loading. As partially described by Okuda (7), the creatinine k' clearly increases with the decrease in salt concentration for weakly bonded supports but not markedly for highly bonded supports.

Using the method described by Blanchard (8), serum was deproteinized before injection to prevent a deterioration of the column performance. The precipitation with trichloroacetic acid removed more than 98% of the proteins with a creatinine recovery of 95 ± 2%. Fig. 1 shows a typical chromatogram of rat serum using a reversed phenyl phase.

TABLE 1

Variations of Creatinine k' with Carbon loading and Salt Concentrations

Bonded phase	Carbon loading %	Creatinine k'		
		Am. Ac. 3.10^{-2} M	Am. Ac. 3.10^{-3} M	Am. Ac. 3.10^{-4} M
C_8	6	1	1.9	2
C_8	12.6	0.4	0.4	0.5
C_{18}	9.4	1.4	1.9	2.4
C_{18}	22.9	0.05	0.05	0.07
phenyl	4.6	1.6	1.9	2.5
phenyl	12.4	1.8	2.9	4.7

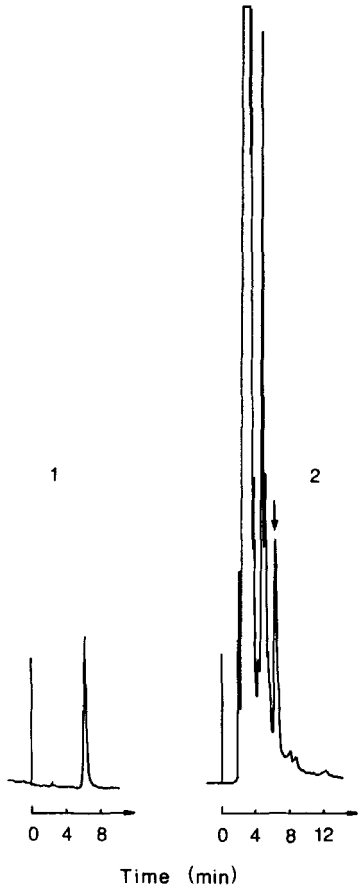

FIGURE 1. Reversed phenyl phase HPLC chromatograms of standard creatinine (1) and rat serum (2) . Mobile phase : aqueous ammonium acetate 3.10^{-2}M 1 ml/min. Standard solution of creatinine : 2.02 µg/ml in eluant. Creatinine retention time : 6 min.

In Vivo Study

Table 2 shows the mean values of the serum creatinine contents both colorimetrically and chromatographically determined in groups of rats exposed, for a 4h period, to 495, 926, 1502 and 1738 ppm of NM. The colorimetrically measured creatininemia of controls exceeded by 3 mg/l that measured by means of the HPLC method. In the NM-exposed rats, colorimetrically estimated creatininemia increased as a function of the exposure to NM without any other biochemical sign of nephrotoxicity (results not detailed here). The highest level of exposure to NM vapors was responsible for a rise in the amount of serum creatinine to 53.0 ± 2.7 mg/l as against 5.1 ± 0.2 mg/l in the control group.

In contrast, the HPLC-assayed creatinine contents of the same blood samples from NM-exposed rats were not significantly different from those of controls whatever the level of exposure to NM.

As seen in Table 3, a dose-dependent increase of creatinine as the result of $HgCl_2$ injection is evidenced by both analytical procedures. A regression analysis (Fig. 2) shows the strong relationship between HPLC and the Jaffé reaction ($r = 0.999$). The colorimetric values exceeded those resulting from HPLC by approximately 1 to 3 mg/l.

In Vitro Study

As shown in Fig. 3, the addition of NM to rat sera in concentrations ranging from 4 to 82 mg/l gave rise to a dose-dependent increase in the colorimetrically measured creatinine contents. In contrast the creatinine values resulting from HPLC

TABLE 2

Effects of NM on Serum Creatinine Content estimated by the Jaffé Reaction and HPLC in rats

Nitromethane exposure level	Creatinine content mg/l[a] (Jaffé reaction)		Creatinine content mg/l[a] (HPLC)	
ppm	Control	Exposed	Control	Exposed
495	5.7 ± 0.1	6.5 ± 0.4	2.1 ± 0.1	2.4 ± 0.2
926	5.5 ± 0.1	13.2 ± 3.0*	1.6 ± 0.1	1.8 ± 0.1
1502	5.7 ± 0.2	35.1 ± 5.6*	2.2 ± 0.2	2.6 ± 0.1
1738	5.1 ± 0.2	53.0 ± 2.7*	1.6 ± 0.1	1.9 ± 0.1

[a] Data are means ± SE for 5 rats
Values are significant * $P<0.05$ (Mann-Whitney test) versus control group.

TABLE 3

Effects of Hg Cl_2 on Serum Creatinine Content estimated by the Jaffé Reaction and HPLC in rats

$HgCl_2$ concentration mg/kg	Creatinine content[a] mg/l (Jaffé reaction)	Creatinine content[a] mg/l (HPLC)
0 ▫ Controls	5.5 ± 0.1	1.7 ± 0.04
2	12.5 ± 2.3	8.7 ± 2.2
4	21.8 ± 3.7*	19.5 ± 4.0 *
5	29.3 ± 3.1*	27.1 ± 3.1 *

[a] Data are means ± S.E. for 5 rats.
Values are significant * $P<0.05$ (Mann-Whitney test) versus control group.

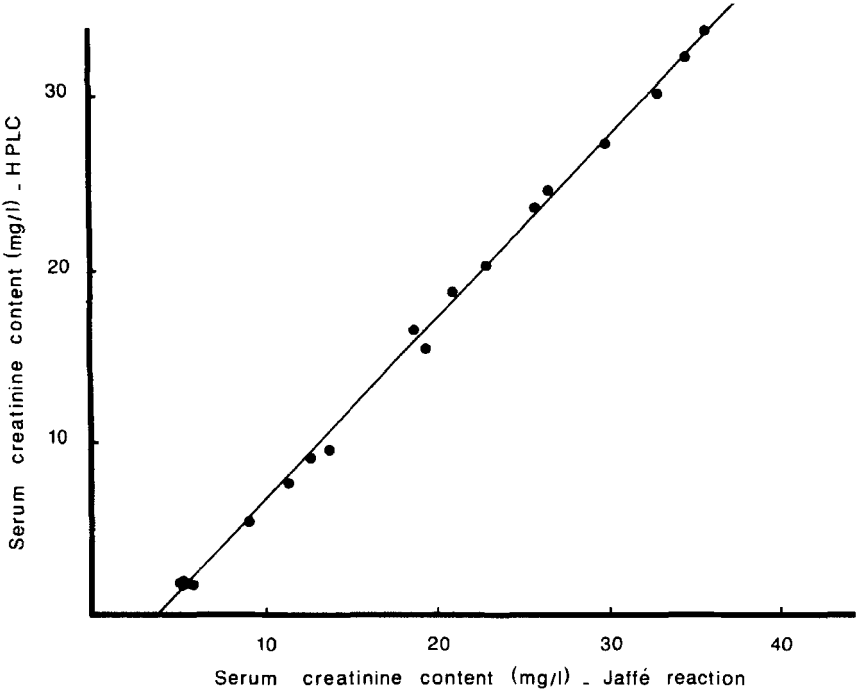

FIGURE 2. Correlation between serum creatinine contents measured by the Jaffé reaction and HPLC. The individual data were obtained from $HgCl_2$-treated rats.

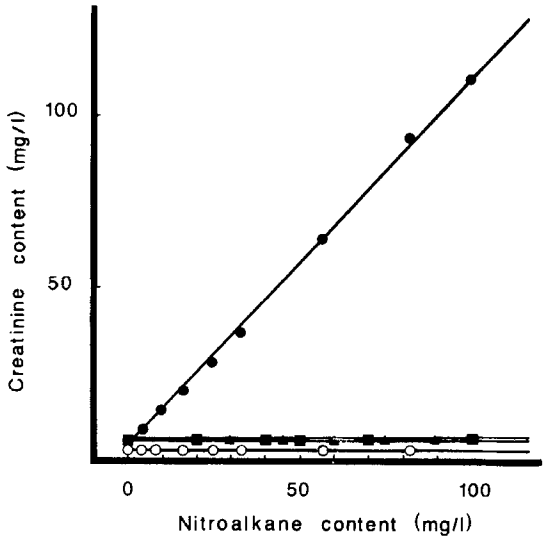

FIGURE 3. The influence of the addition of nitroalkanes on serum creatinine content performed by the Jaffé reaction (● NM, ■ NE, ▲ NP) or by HPLC (○ NM).

analysis of the same NM-dosed sera did not differ from those of controls. It is apparent that the colorimetrically measured creatinine always exceeded that measured chromatographically, the excess being approximately 2 to 3 mg/l.

Sera supplemented either with NE or NP in the dose-range of 30 to 100 mg/l did not develop any above-normal coloured reaction.

DISCUSSION

The measurement of creatinine in serum is done routinely in experimental toxicology, as in clinical situations, to determine the state of renal function, especially glomerular integrity.

This work indicates that colorimetry is a falsely responding procedure for analyzing the sera of NM-exposed rats or NM-supplemented sera while HPLC is ideal for this purpose. The use of phenyl phases offers the best compromise between short analysis time and good selectivity of separation at 3.10^{-2} M ammonium acetate concentration. Furthermore, the low variability of k' with high and weak loading allows both phenyl phases to be used indifferently. In addition to other factors known to influence creatinine k', such as pH (13), salt concentration (7) and the history of the column, the results of this work demonstrate influence of the carbon loading and the nature of bonded phases. It would be interesting to consider subsequently the influence of accessible silanol groups. Furthermore, the lower level of creatinine in rats than in humans necessitates the most sensitive of methods. Trichloroacetic acid precipitation, of anionic type, inducing a weaker dilution (serum/precipitant volumes of 1/0.2) was consequently preferred to organic solvent precipitation (serum/precipitant volumes of 1/1.5).

Many substances have been reported to interfere with the Jaffé reaction through chemical mechanisms (1). However, the presence of both nitroethane and 2-nitropropane in colorimetrically analyzed sera does not allow any above-normal creatinine content to be determined. Therefore, it is suggested that the concentration-dependent intensity of the coloured reaction developed by NM-exposed rat sera or by NM-supplemented sera could be ascribed to the peculiar reactivity of this chemical. To explain this

difference in reactivity, reference may be made to the inductive effect of the alkyl group substituants which control the rate of proton loss from nitroalkanes just as they do from ketones (14,15). The resulting nitronate anion of NM might be expected to form a coloured adduct in a manner similar to that of creatinine.

Our results confirm the existence of a close link between the values obtained through each method for a wide range of in vivo and in vitro creatinine contents, as has been reported in clinical studies (6, 7). However, as previously shown (7, 4), the colorimetric method, moderately but unvaryingly, overestimates HPLC-determined creatinine contents. Such a discrepancy may be due to the lack of specificity of the Jaffé reaction which also measures pseudochromogens in addition to creatinine (16).

The occurrence of NM interference with the Jaffé reaction must be kept in mind in both occupational and experimental toxicology. It may be advisable in future to determine a strategy for detecting interference by industrial chemicals with commonly used laboratory tests and to encourage the development of more specific methods of analysis, such as HPLC.

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